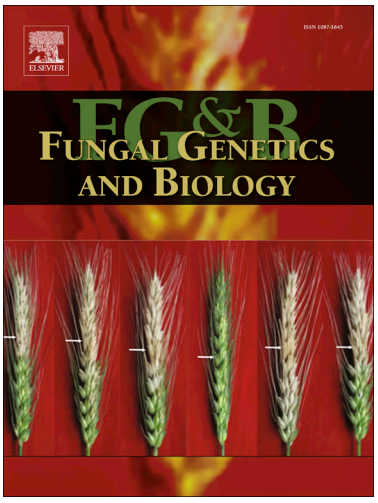

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Roles of the *Skn7* response regulator in stress resistance, cell wall integrity and GA biosynthesis in *Ganoderma lucidum*

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Abstract

The transcription factor *Skn7* is a highly conserved fungal protein that participates in a variety of processes, including oxidative stress adaptation, fungicide sensitivity, cell wall biosynthesis, cell cycle, and sporulation. In this study, a homologous gene of *Saccharomyces cerevisiae Skn7* was cloned from *Ganoderma lucidum*. RNA interference (RNAi) was used to study the functions of *Skn7*, and the two knockdown strains *Skn7i-5* and *Skn7i-7* were obtained in *G. lucidum*. The knockdown of *GlSkn7* resulted in hypersensitivity to oxidative and cell wall stresses. The concentrations of chitin and *β*-1,3-glucan distinctly decreased in the *GlSkn7* knockdown strains compared with those of the wild type (WT). In addition, the expression of cell wall biosynthesis related genes was also significantly down-regulated and the thickness of the cell wall also significantly reduced in the *GlSkn7* knockdown strains. The intracellular reactive oxygen species (ROS) content and ganoderic acids biosynthesis increased significantly in the *GlSkn7* knockdown strains. Interestingly, the level of intracellular ROS and the content of ganoderic acids decreased after N-acetyl-L-cysteine (NAC), an ROS scavenger, was added, indicating that *GlSkn7* might regulate ganoderic acids biosynthesis via the intracellular ROS

level. The transcript level of *GlSkn7* were up-regulated in osmotic stress, heat stress and fungicide condition. At the same time, the content of ganoderic acids in the *GlSkn7* knockdown strains also changed distinctly in these conditions. Overall, *GlSkn7* is involved in stress resistance, cell wall integrity and ganoderic acid biosynthesis in *G. lucidum.*

Keywords: *Ganoderma lucidum*, Cell wall integrity, Reactive oxygen species, Ganoderic acids

1. Introduction

Ganoderma lucidum (Leyss.:Fr.) Karst is one of the best-known medicinal macro-fungi in the world. Because of this fungus's multiple medicinal properties, including anti-tumor, anti-hypertensive, antiviral and immunomodulatory activities (Calvino et al., 2010; Yue et al., 2010), *G. lucidum* has been extensively utilized in China and Southeast Asia for centuries to treat various human diseases, promote longevity, and improve vigor (Paterson, 2006). Numerous studies have suggested that ganoderic acids (GAs) are among the main types of active components of this fungus and are likely to be responsible for its remarkable therapeutic effects in various human diseases (Jedinak et al., 2011; Joseph et al., 2011). A substantial amount of research has been conducted to improve the content of GAs (Li et al., 2016; You et al., 2017). Since GAs are secondary metabolites, their biosynthesis is easily affected by environmental stresses, therefore, the mechanism of the regulation of the GA biosynthesis by

environmental factors is a highly active area of research (Zhang et al., 2016). Further research has shown that the signal molecules known as reactive oxygen species (ROS) might be a key factor in regulating GA biosynthesis (Ren et al., 2017). However, the homeostasis of ROS is a complex system that is regulated by multiple genes (ROS generation, clearance, response factor). Thus, additional research is required to study the responses of these genes to environmental changes and their effects on the balance of ROS.

Skn7 is a response regulator that is located downstream from the two-component signal transduction pathway that is one of the key signaling pathways in bacteria, yeasts, plants, and filamentous fungi, enabling organisms to adapt to various environmental stresses (Koretke et al., 2000; Santos and Shiozaki, 2001). As a highly conserved stress-responsive transcription factor, *Skn7* is involved in a variety of processes, including oxidative stress adaptation, fungicide sensitivity, cell wall biosynthesis, cell cycle, and sporulation (Brown et al., 1993; Brown et al., 1994; Bouquin et al., 1999; Tao et al., 1999). The most important function of *Skn7* is resistance to environmental stressors that have been extensively documented in many species (Chen et al., 2012; Shalaby et al., 2014). In addition, *Skn7* is also considered to be an indispensable part of the ROS system and to maintain the homeostasis of ROS. Under oxidative stress, *Skn7* can form a complex with the stress responsive transcription regulator *Yap1*, and the complex is necessary for the normal induction of oxidative stress response (OXR) genes (Lu et al., 2003; Lu et al., 2004; He and Fassler, 2005; Fassler and West, 2011). Although

Skn7 function and its interactions have been extensively studied in ascomycetes, very few studies have been made in basidiomycetes except *Cryptococcus neoformans* (Bahn et al., 2006). In general, these *Skn7* orthologues have been observed to be associated with the resistance to various stress conditions. However, the exact functions of *Skn7* vary among fungi and have not been reported to date in macro-basidiomycetes.

Collectively, *Skn7* serves an important function to resist environment stresses and maintain the balance of the ROS system. To study the functions of *Skn7* in *G. lucidum*, the *Skn7* gene was cloned, and silenced strains were constructed utilizing the RNA interference (RNAi) method described in this study. We observed that *GlSkn7* knockdown strains were more sensitive to oxidative stress and cell wall stress than the wild type and that the concentrations of chitin and β-1,3-glucan and the thickness of the cell wall were also reduced significantly. when the expression level of the *GlSkn7* gene decreased, the concentrations of the intracellular ROS and GAs increased significantly. We also observed that *GlSkn7* might regulate the biosynthesis of GAs by influencing the content of intracellular ROS. In addition, the transcript level of *GlSkn7* were up-regulated and the content of GAs in the *GlSkn7* knockdown strains also changed distinctly in osmotic stress, heat stress and fungicide condition. Taken together, our results showed that *GlSkn7* could be implicated in stress resistance, cell wall integrity, and GAs biosynthesis and indicated that *GlSkn7* might regulate the biosynthesis of GAs via the intracellular ROS level in *G. lucidum*.

2. Materials and Methods

2.1 Strains and growth conditions.

Escherichia coli DH5α was grown in Luria Broth (LB) medium that contained kanamycin (50 μg mL^{-1}) or ampicillin (100 μg mL^{-1}) to amplify the plasmid. The *G. lucidum* ACCC53264 strain (dicaryon) was obtained from the Agricultural Culture Collection of China. The WT (wild-type), CK (empty-vector controls), and *GlSkn7* knockdown strains were cultured at 28 °C for 7 days in CYM medium (2% glucose, 1% maltose, 0.05% $MgSO_4.7H_2O$, 0.2% yeast extract, 0.46% KH_2PO_4 and 0.2% tryptone), which were in stationary phase for additional experiments.

2.2 Gene cloning and bioinformatics sequence analysis

The entire sequence of *G. lucidum Skn7* (*GlSkn7*) was obtained by the polymerase chain reaction (PCR) utilizing *G. lucidum* cDNA as templates with the primers listed in Table S1. The fragments were inserted into the pMD18-T vector (TaKaRa, Dalian, China) for sequencing. The National Center for Biotechnology Information (NCBI) was used for BLAST similarity searches. The ORF and exon/intron positions were inferred from comparisons of the genomic and cDNA sequences. The theoretical molecular weight of the protein was predicted utilizing the online ExPASY tool (http://expasy.org/tools/pi_tool.html). The conserved motifs were determined from the NCBI Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd). Multiple sequence alignments were performed utilizing the DNAMAN program. The JASPAR database (http://jaspardev.genereg.net/) was

used to analyze the putative *GlSkn7* binding site.

2.3 Construction of RNAi plasmids and strains

The construction of the RNAi vector and the empty-vector control (CK), the transformation of the *G. lucidum* strain, and the random screening of the transformants were performed as described previously (Mu et al., 2012). The dual-promoter-silencing vector pAN7-dual that is driven by the glyceraldehyde-3-phosphatedehydrogenase (*gpd*) promoter and the 35S promoter was used to suppress the expression of *Skn7*. The coding region of *Skn7* was amplified by PCR utilizing *G. lucidum* cDNA as a template with the primers listed in Table S1. The *Skn7* fragment was doubly digested with *Kpn*I and *Spe*I and was inserted into the plasmid pAN7-dual after *Kpn*I/*Spe*I restriction digestion. This plasmid was used to transform *G. lucidum* by electroporation as described by Mu (Mu et al., 2012).

2.4 Real-time PCR analysis of gene expression

Total RNA was extracted utilizing an RNA Isolation Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The levels of gene-specific messenger RNA (mRNA) expressed by the WT and *GlSkn7* knockout strains were evaluated by quantitative real-time PCR as described previously (Mu et al., 2014). In addition, the expression patterns of the *Skn7* genes in mycelia grown in solid cultivation were analyzed utilizing the same method. The primers are displayed in Supplementary Table 1.

2.5 Stress sensitivity assays

A 6-mm-diameter hyphal tip plug of all of the strains was cultured on CYM plates supplemented with 8 mM H_2O_2, 3.5 mM KO_2 and cell wall inhibitors (0.01% SDS and 2 mg mL^{-1} Congo red) for 7 days to assay oxidative and cell wall stresses. Radial hyphal growth was measured to observe the tolerance of all of the strains to stress conditions. All of the strains were generated in triplicate, and the experiment was repeated twice.

2.6 Quantification of *β*-1, 3-glucan and cell wall chitin

All of the strains were inoculated on CYM plates for 7 days to quantify the concentrations of *β*-1,3-glucan and cell wall chitin. The concentration of *β*-1, 3-glucan was examined utilizing the aniline blue assay as described by Fortwendel (Fortwendel et al., 2009). Chitin assays were performed as described previously (Lehmann and White, 1975). The detailed experimental process was reported in our previous study (Zhang et al., 2017).

2.7 Ultrastructural observation of the cell wall.

Transmission electron microscopy (TEM) were performed for ultrastructure observations of the WT and *GlSkn7* knockdown strains. Fungal 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.

2.8 ROS detection assay

The WT, CKs and *GlSkn7* knockdown strains were stained with DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate). ROS were detected utilizing a Zeiss Axio Imager A1 fluorescence microscope, the ZEN liFte (Zeiss software) was used to monitor the quantity of the fluorescence intensity, and the detailed method was described previously (Mu et al., 2014). In addition, the concentrations of H_2O_2 in the samples tested were quantified utilizing a commercial hydrogen peroxide assay kit (Nanjing Jiancheng Bioengineering Institute, China).

2.9 Quantification of ganoderic acid levels

Ganoderic acids were extracted and quantified as described previously (Shi et al., 2015).

3.0 Statistical analysis

The data from at least three independent sample measurements was averaged to ensure that the trends and relationships observed in the cultures were reproducible. The mean values of each experiment were expressed as the mean ± SD. Appropriate statistical tests were used in each figure Student's *t*-test was used to compare two samples. A Duncan's multiple range test was used for multiple comparisons. A P-value of < 0.05 was considered to be significant.

3. Results

3.1 Cloning and sequence analysis of the *Skn7* gene

Based on the amino acid similarity to *S. cerevisiae Skn7* (NCBI taxid:AAC48911.1), only the gene *GlSkn7* (GenBank accession number:MF807226) was screened from the *G. lucidum* genome database

(http://www.herbalgenomics.org/galu/). The *Skn7* genomic sequence was 2,471 bp and contained six introns. The length of the *Skn7* cDNA was 1,716 bp, and predictions indicated that this cDNA encodes a protein of 571 amino acids with a molecular mass of 63.74 kDa and an isoelectric point of 5.59. Analysis of the predicted amino acid sequence of *GlSkn7* identified two conserved domains: the heat stress transcription factor (HSF)-type DNA-binding domain and the signal receiver domain that can be easily detected in multiple sequence alignments commonly found in the *Skn7*-like proteins (Fig. 1A and B). The amino acid sequence of the HSF domain of *GlSkn7* shows 84.34% identity with that of the *Gloeophyllum trabeum Skn7* protein, and the signal receiver domain of *GlSkn7* is also conserved that h as 84.21% amino acid sequence similarity with that of the *Gloeophyllum trabeum Skn7* protein. These results implied that the *GlSkn7* gene that was cloned belongs to the *Skn7* family.

3.2 Construction of G. lucidum *Skn7* knockdown strains

To explore the function of *Skn7* in *G. lucidum*, we constructed a *Skn7*-silencing vector with a dual promoter system described previously (Mu et al., 2012). The vector that was constructed as shown in Fig. 2A was denominated pRNAi-*GlSkn7*. The vector pAN7-ura3-dual was used as an empty vector control. All of the vectors were transformed into the WT *G. lucidum* by electroporation, and the positive transformants grew on CYM plate media that contained hygromycin. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was utilized to detect the transcription level of *Skn7* in the selected mutant strains. Thirty positive transformants were selected, and eight of them had a higher

silencing efficiency, with their inhibition efficiency approximating 75%-95%. *Skn7i-5* and *Skn7i-7* strains were randomly selected for further analysis, and the expression of the *Skn7* gene in mutants was decreased by 88% and 90%, respectively, compared with the WT (Fig. 2B).

3.3 Effect of the *GlSkn7* on the accumulation of ROS in *G. lucidum*

Previous studies have shown that *Skn7* can regulate key genes associated with antioxidant systems, thereby influencing the homeostasis of the intracellular ROS (He et al., 2009). To investigate the roles of *GlSkn7* in the balance of ROS, we analyzed the concentrations of ROS in the *GlSkn7* knockdown strains. The compound 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) was utilized as an ROS fluorescent probe. As shown in Fig. 3A and B, the fluorescence intensity of the *Skn7i-5* and *Skn7i-7* knockdown strains was significantly higher than that of the WT, and the ROS fluorescence value was up-regulated by approximately 1.78-fold and 1.82-fold compared with the WT, respectively. To study the change in the ROS level, the intracellular H_2O_2 concentration was also quantitatively measured in the *Skn7*-silenced strains (Fig. 3C) and was up-regulated by approximately 73.7% and 76.4% in the *Skn7i-5* and *Skn7i-7* knockdown strains, respectively. The trend of this change in the H_2O_2 concentration was consistent with that of the fluorescence probe resulting from the *GlSkn7* knockdown strain.

The homeostasis of ROS is synergistically regulated by ROS generation and scavenging systems; therefore, we examined the detectable gene expression of nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase (*noxa, noxb, noxr*), the main source of ROS, and enzymes related to the ROS scavenging system, including superoxide dismutase (SOD), catalase, (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), thioredoxin (TRX2), and thioredoxin reductase (TRR1). Our results showed that there were no significant effects on the transcript levels of NADPH oxidase in the *GlSkn7* knockdown strains compared with those of the WT strain (Fig. 4A, B and C). However, the transcript levels of these antioxidant genes were significantly down-regulated in the *GlSkn7* knockdown strains (Fig. 4D~L). It was reported that *Skn7* could bind to promoters of some antioxidant genes in *S. cerevisiae* (He and Fassler 2005). To examine whether these antioxidant genes of *G. lucidum* have putative *GlSkn7* response elements in their promoters, the JASPAR database (http://jaspardev.genereg.net/) was used to analyze the putative *GlSkn7* binding site. As shown in the Fig. S1, a conserved site, GGC(C/G)(A/G), was present in the promoter regions of all these genes. The motif is highly similar to the yeast *Skn7* binding site GGC(C/G)(A/G) (MacIsaac et al., 2006), suggesting that is the putative binding site of *GlSkn7*. As a second oxidative stress response transcription factor, *Yap1* can forms a complex with *Skn7* to regulates the expression of most of the antioxidant genes (He and Fassler, 2005; Lu et al., 2003). So we representatively selected some genes regulated by *Yap*1 in *S. cerevisae* (Lee et al., 1999) and examine these homologous genes level of expression in *GlSkn7* knockdown strains. Our results showed the transcript levels of Yap1-dependent and Skn7-dependent antioxidant genes were also significantly down-regulated in the *GlSkn7*

knockdown strains (Fig. S2). In addition to mediating the interaction with *Yap1*, the *Skn7* receiver domain is responsible for the interaction with the Ran binding protein, *Mog1* (Lu et al., 2004). So we selected some genes regulated by *Mog1* (Stochaj et al., 1998; Ma et al., 2007) and examine these homologous genes level of expression in *GlSkn7* knockdown strains. Our results showed that there were no significant effects on the transcript levels of *Mog1*-regulated genes in the *GlSkn7* knockdown strains compared with those of the WT strain (Fig. S3). Overall, these results implied that the *GlSkn7* knockdown led to an increase in the intracellular ROS in *G. lucidum* by down-regulating the expression of most of the antioxidant genes.

3.4 Involvement of *GlSkn7* in the resistance to oxidative and cell wall stresses

The change of intracellular ROS is related to a variety of types of signal transduction and further regulates the cell's response to various environment stresses. When *GlSkn7* was knocked down, the levels of ROS were changed. To investigate the role of *GlSkn7* in resisting oxidative stress, the WT, CKs and *GlSkn7* knockdown strains were cultured on CYM plate media containing exogenous 8 mM hydrogen peroxide (H_2O_2) and 3.5 mM potassium superoxide (KO_2). Hydrogen peroxide and potassium superoxide are the most typical oxidants in cells under oxidative stress conditions. As shown in Fig. 5A and B, the relative growth inhibition rate increased by approximately 101% and 106% in the *Skn7i*-5 and *Skn7i*-7 strains, respectively, compared to the WT strain after the addition of 8 mM exogenous H_2O_2. The relative growth inhibition rate increased by approximately 111% and 108% in the *Skn7i*-5

and *Skn7i-7* strains, respectively, after the addition of 3.5 mM exogenous KO_2. These results showed that the *GlSkn7* knockdown strains were more sensitive to oxidative stress than the WT, thereby implying that *GlSkn7* might play an important role in oxidative stress resistance.

To explore whether *GlSkn7* also participates in other stress conditions, the sensitivity of *GlSkn7* to cell wall stress was tested. The WT, CKs and *GlSkn7* knockdown strains grew on CYM plate media containing the cell wall interfering substances 0.01% sodium dodecyl sulfate (SDS) and 2 mg mL^{-1} Congo red (CR) to measure the ability of their growth. Compared with the WT strain, the relative growth inhibition rates increased by approximately 54% and 53% in the *Skn7i-5* and *Skn7i-7* strains, respectively, after the addition of 0.01% SDS. After the addition of 2 mg mL^{-1} CR, these rates increased by approximately 27% and 30% in the *Skn7i-5* and *Skn7i-7* strains, respectively (Fig. 5C and D). These results showed that the sensitivity of the *GlSkn7* knockdown strains increased significantly compared with that of the WT during cell wall stress, indicating that *GlSkn7* might participate in the response to this type of stress.

3.5 Chitin and *β*-1,3-glucan contents decreased in the *GlSkn7* knockdown strains

Since the *GlSkn7* mutant strains exhibit increased sensitivity to cell wall stress, we measured the concentrations of chitin and *β*-1,3-glucan that are the major components of the cell wall in all of the strains tested. As shown in Fig. 6A and B, the concentration of chitin in the *Skn7i-5* and *Skn7i-7* knockdown strains decreased by 55.8% and 51.0% compared with the WT, respectively. The

concentration of *β*-1,3-glucan in the *Skn7i-5* and *Skn7i-7* knockdown strains decreased by 45.9% and 46.5%, respectively.

In addition, we examined the level of transcription of the cell wall biosynthetic genes of *G. lucidum*. We identified eight putative chitin synthase coding genes (GL30737, GL31550, GL25613, GL28060, GL30799, GL27969, GL18134 and GL15273) and three putative glucan synthase-coding genes (GL20535, GL24465 and GL24554) involved in the synthesis of chitin and glucan, respectively, from the genome of *G. lucidum*. As shown in Fig. 6C and D, the expression of these genes was significantly down-regulated in the *GlSkn7* knockdown strains compared with those in the WT strain. These results implied that *GlSkn7* might influence the synthesis of the glucan and chitin in *G. lucidum*.

3.6 The thickness of the cell wall decreased in *GlSkn7* knockdown strains

To further detect cell wall changes when the expression level of the *GlSkn7* gene decreased, we detected the thickness of the cell wall by TEM. The results showed that the thickness of the cell wall in *Skn7i-5* and *Skn7i-7* knockdown strains was decreased 54.8% and 61.9%, respectively, compared with the WT, which were consistent with the decreased the content of chitin and *β*-1,3-glucan (Fig. 7A and B). Taken together, these results indicate that *GlSkn7* might be involved in the cell wall integrity.

3.7 *GlSkn7* influences ganoderic acids biosynthesis

GAs are important secondary metabolites of *G. lucidum*. The concentrations of the metabolites in these strains were analyzed to determine whether *GlSkn7* influences the biosynthesis of GAs. As shown

in Fig. 8A, the concentrations of the GAs in the *Skn7i-5* and *Skn7i-7* knockdown strains increased by 52.1% and 55.9%, respectively, compared with the WT. To study the effect of *GlSkn7* on GA biosynthesis more thoroughly, the expression of three key enzyme genes in the biosynthesis of GAs was analyzed. Hmgr (3-hydroxy-3-methylglutaryl coenzyme A reductase), sqs (squalene synthase), and osc (lanosterol synthase) are the key enzymes in the GA biosynthetic pathway. The transcript levels of the *hmgr* gene were up-regulated by approximately 56% and 52%; the transcript levels of the *osc* gene were up-regulated by approximately95% and 93%, and the transcript levels of the *sqs* gene were up-regulated by approximately 49% and 53%% in the *Skn7i-5* and *Skn7i-7* strains, respectively, compared with those in the WT strain (Fig. 8B, C and D). To study whether *GlSkn7* can reduce GAs biosynthesis along the different phases of the mycelia cultures, all the tested strains were cultured respectively for 3d, 4d, 5d and 6d in liquid CYM medium to detect the content of GAs. As shown in Fig. S4, the content of GAs in *GlSkn7* knockdown strains was always significantly increased compared with WT cultured respectively for 3d, 4d, 5d and 6d. These results suggested that *GlSkn7* might be involved in the regulation process of GAs biosynthesis.

3.8 *GlSkn7* regulates the accumulation of GAs via intracellular ROS.

When *GlSkn7* was knocked down, the amounts of ROS and H_2O_2 clearly increased, and the amount of GAs exhibited the same trend compared with the WT. To determine whether *GlSkn7* affects the biosynthesis of GAs via ROS, the ROS scavenger 5 mM N-acetyl cysteine (NAC) was added to the

CYM solid agar medium. As shown in Fig. 9A, B and C, the amounts of ROS and H_2O_2 both decreased to the WT levels in *GlSkn7* knockdown strains after the addition of NAC.

To determine whether the increase of the GAs content in the *Skn7*-silenced strains was due to the increased ROS content, 5 mM NAC was added to the CYM liquid culture medium, and the concentrations of the GAs were measured. As expected, after adding 5 mM NAC to the *GlSkn7* knockdown mutants, the concentrations of GAs decreased significantly and did not differ significantly from the WT (Fig. 10A). The three key genes for the GAs biosynthetic pathway (*hmgr*, *sqs*, and *osc*) also exhibited a similar tendency (Fig. 10 B, C and D). These results imply that *GlSkn7* can regulate the ROS levels, which subsequently then influence GAs biosynthesis by affecting the gene expression of the key enzymes in this process.

3.9 *GlSkn7* influences GAs biosynthesis in osmotic stress, heat stress and fungicide condition.

It was reported that the functions of *Skn7* was different in the process of stress response in other fungi (He and Fassler, 2005). To study *GlSkn7* how to function in osmotic stress, heat stress and fungicide conditions, we examined the transcript level of *GlSkn7* in these conditions. As shown in the Fig. 11A, the transcript level of *GlSkn7* were up-regulated by approximately 116%, 113%, 60.7% and 61.6% in 1M NaCl, 1M KCl, 1M Sorbitol and 1M Mannitol condition respectively. These results suggested *GlSkn7* might play a role in osmotic condition. However, there was no significant effects on the transcript levels of *GlSkn7* in 1M NaCl and 1M KCl condition. The result implied that *GlSkn7*

might not respond to sodium stress, but to osmotic condition per se (Bahn et al., 2006). In addition, the transcript level of *GlSkn7* were up-regulated by approximately 84.7%, and 81.3% ,respectively, in heat stress and 100 ug/ml fludioxonil condition. These results also indicated *GlSkn7* might play a role in heat stress and fungicide resistance.

GAs biosynthesis is easily influenced by stress factors. To study *GlSkn7* how to regulate GAs biosynthesis, the content of GAs in all tested strains were measured. In 1M NaCl and 1M KCl conditions, the content of GAs in *GlSkn7* knockdown strains were significantly increased and the content of GAs in WT strains were significantly decreased. In 1M Sorbitol and 1M Mannitol condition, the content of GAs in *GlSkn7* knockdown strains were no significant change and the content of GAs in WT strains were significantly decreased (Fig. 11B). These results indicated *GlSkn7* might negatively regulate GAs biosynthesis in osmotic stress. In heat stress, the content of GAs in *Skn7i-5 and Skn7i-7* knockdown strains increased approximately 63% and 64% and the content of GAs in WT increased approximately 62% (Fig. 11C). The results implied that *GlSkn7* might have no effect on GAs biosynthesis in heat stress. As shown in Fig. 11D, the content of GAs in *GlSkn7* knockdown strains significantly decreased and the content of GAs in WT were no significant change in fungicide condition. The results suggested that *GlSkn7* might positively regulate GAs biosynthesis in fungicide condition. In general, these results indicated *GlSkn7* might have on different effects on GAs biosynthesis in osmotic stress, heat stress and fungicide condition.

4. Discussion

Two-component signaling is critical to the stress adaptation of such fungi as *Saccharomyces cerevisiae* and *Candida albicans* (Hohmann, 2002; Singh et al., 2004). *Skn7*, as an effector of the two-component signaling system, has been shown to be associated with many aspects of this adaptation. In this study, we first constructed *GlSkn7* knockdown strains to characterize the functions of the *GlSkn7* gene in macro-basidiomycetes. We demonstrated its various functions in physiological processes, including resistance to stresses, regulation of cell wall integrity, and secondary metabolism in *G.lucidum*.

All organisms, including fungi, require resistance to abiotic stresses in order to survive adverse environmental conditions. It has been reported that ROS, as a signaling molecule, is involved in the process of resistance to stress conditions (Schieber and Chandel, 2014). We found that the amount of ROS in mutants rises distinctly, while the gene expression of the related antioxidant enzymes is reduced in the mutants. And we also found a putative *GlSkn7* binding site, GGC(C/G)(A/G), in promoters of these antioxidant genes. This finding suggested that *Skn7* could regulate the ROS homeostasis by affecting the expression of antioxidative genes. In *Cochliobolus heterostrophus*, the loss of *Skn7* also led to a reduction in the gene expression of the related ROS scavenging enzymes (Shalaby et al., 2014). And a similar *MrSkn7* binding site, GGCC(A/G), was also present in the promoter regions of target genes in *Metarhizium robertsii* (Shang et al., 2015). In addition, we found

that the *GlSkn7* knockout mutants became more sensitive to H_2O_2 compared with WT. During oxidative stress, many fungi exhibit a phenotype similar to that of the *Skn7* knockout mutants resulting in increased sensitivity to H_2O_2, that is consistent with our results (Coenjaerts et al., 2006; Chenet al., 2012; Shalaby et al., 2014; Yang et al., 2015). These results show that *Skn7* can regulate ROS homeostasis and be involved in the resistance to oxidative stress. In addition, the sensitivity of *Skn7* to oxidative stress may also be related to the decrease in the gene expression levels of enzymes related to the ROS scavenging system in the *Skn7* mutant strains.

Cell wall integrity is crucial for the growth, pathogenesis, and survival of microorganisms. In this study, we observed that the *GlSkn7* knockdown strains exhibited increased sensitivity to SDS and CR, a decrease in the amounts of chitin and β-1,3-glucan, and the down-regulation of the expression of genes related to cell wall biosynthesis. We further observed that the thickness of the cell wall in the *GlSkn7* knockdown strains significantly reduced by TEM. Similar results have been reported in other fungi. For example, *Aspergillus fumigatus Skn7* mutants exhibited increased sensitivity to sodium dodecyl sulfate (SDS) (Lamarre et al., 2007), and *Cryptococcus neoformans Skn7* mutants had enhanced resistance to the chitin-binding compound Congo Red (CR) (Wormley et al., 2005). Morphogenetic defects noted in *Candida lusitaniae* might also reflect cell wall remodeling defects in the absence of Skn7 (Ruprich-Robert et al., 2008). In addition, the ability of *Skn7* to bind to the promoter of the OCH1 gene that encodes an α-1,6-mannosyltransferase has also been reported in yeast (Li et al., 2002). These

results suggest that *Skn7* is involved in the maintenance of cell wall integrity in fungi. However, the mechanism that *Skn7* utilizes to participate in this process is not very clear and merits additional research.

The fact that *Skn7* regulates the biosynthesis of fungal secondary metabolites has not been widely reported to date. In our study, we observed that the concentration of GAs and the expression of key genes related to GAs biosynthesis increased significantly in the *GlSkn7* knockdown strains and demonstrated that *Skn7* is involved in the process of secondary metabolic biosynthesis in *G. lucidum*. There was a similar report that *Botrytis cinerea Skn7* was associated with the regulation of the transcription level of the genes related to ergosterol biosynthesis (Yang et al., 2015). Further study on the mechanism that *Skn7* utilizes to affect the biosynthesis of GAs may confirm the research suggesting that this process may be regulated by the intracellular ROS system. Our previous works found that the influences of genes involved in ROS scavenge system (Li et al., 2015) and ROS generation system (Mu et al., 2014) on biosynthesis of GAs were different. In some conditions the ROS signal is positively correlated with the GAs biosynthesis (Mu et al., 2014), but in other conditions, the ROS signal is negatively correlated with the GAs biosynthesis (Li et al., 2015). Hence, we analyzed the functions of other ROS related genes in regulating the biosynthesis of GAs. *Skn7*, as a ROS response factors, can help us to further enrich the study of ROS system. At the same time, *Skn7* can also participate in stress response and signal transduction network to respond to different environment stress (Lee et al., 2016).

In our study, the transcript level of *GlSkn7* was significantly increased in different stress conditions and we further observed that *GlSkn7* had on different effects on GAs biosynthesis in osmotic stress, heat stress and fungicide condition. The works about *Skn7* can help us to further study the mechanism of ROS change under environmental stress. Studies on *Skn7* found that it could also influence the intracellular ROS level, and the ROS levels has a positively correlation with the GAs synthesis. Taken together, these results explain that the ROS level is involved in the regulation of the GA biosynthesis. In *S cerevisiae*, the mechanism that *Skn7* utilizes to affect the level of intracellular ROS has been clearly reported (He et al., 2009). However, the mechanism by which *GlSkn7* regulates intracellular ROS homeostasis leading to the change of secondary metabolites needs to be examined in depth to further clarify its function in secondary metabolism.

In summary, the functional analysis of *GlSkn7* revealed its various functions involved in stress resistance, cell wall integrity and GAs biosynthesis in *G. lucidum*. Further evidence showed that *GlSkn7* might influence cell wall integrity by regulating genes related to cell wall biosynthesis. In addition, *GlSkn7* can also regulate intracellular ROS levels, and ROS might influence GAs biosynthesis and the gene expression of three key enzymes involved in GAs biosynthesis. Thus, an investigation of the detailed molecular mechanism of the involvement of *GlSkn7* in the regulation of secondary metabolism in basidiomycetes merits further study.

Acknowledgments

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Figure legends

FIG.1 Amino acid sequence alignments of the conserved domain in *Skn7* from nine different species.

The residues included in the alignment are indicated to the left. The alignment was generated with DNAMAN software. *Cryptococcus neoformans* (AAV36564.1); *Gloeophyllum trabeum* (XP_007863114); *Candida albicans* (XP_716809.1); *Candida orthopsilosis* (XP_003870355); *Candida dubliniensis* (XP_002420333); *Aspergillus clavatus* (XP_001268306); *Aspergillus fumigatus* (XP_001481472.1); *Neurospora crassa* (XP_959541.3); (A) The *HSF*-type DNA-binding domain. (B) The signal receiver domain. Black shading indicates invariant residues, and two additional shades of gray represent at least 80% conservation and at least 60% conservation, respectively.

FIG.2 Detection of *Skn7* transcript levels in the *Skn7*-silenced strains.

(A) In the plasmid, URA3 transcription is driven by the 35S promoter, and the *Skn7* gene is driven by the glyceraldehyde-3-phosphate dehydrogenase promoter. (B) qRT-PCR analysis of the expression of *GlSkn7* in the tested strains. The relative mRNA levels of *GlSkn7* were calculated as the ratio of *GlSkn7* mRNA to endogenous 18S rRNA. The expression level of the *GlSkn7* gene in the WT strain was arbitrarily set to 1. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines (**, $P < 0.01$, Student´s t-test).

FIG.3 Intracellular ROS accumulation was increased in the *GlSkn7* knockdown strains.

(A) The control (WT, CK1 and CK2) and *Skn7*-silenced *G. lucidum* strains were cultured at 28 °C on CYM solid agar medium for 4 days. The change in the ROS levels was measured by DCFH-DA staining. Scale bar = 100 μm. (B) Changes in the ROS fluorescence ratio in the hyphal regions of the control (WT, CK1 and CK2) and *Skn7*-silenced strains. The y-axis represents the ROS fluorescence ratio as measured by CLSM, and the x-axis represents the different strains. The values in each column represent the captured fluorescence ratio in each observation. (C) Measurement of the amount of H_2O_2 in *G. lucidum*. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines (**, $P < 0.01$, Student´s t-test).

FIG.4 Transcript levels of ROS-regulated enzymes related genes in *GlSkn7* knockdown strains.

(A)-(C) Seven-day-old liquid mycelia from the control (WT, CK1 and CK2) and *Skn7*-silenced strains were collected to measure the relative mRNA level of genes in *G. lucidum* that are related to NADPH oxidase (including *nox*a, *noxb*, *noxr*). (D)-(L) The transcript levels of the SOD (including SOD1, SOD2, and SOD4), CAT (including CAT1 and CAT2), GPX, APX, TRR1, and TRX2-related genes were measured in the strains. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines (**, $P < 0.01$, Student´s t-test).

FIG.5 Phenotypic assays of the *Skn*7-silenced strains exposed to oxidative stress and cell wall stress.

(A) *G. lucidum* strains were grown on CYM solid agar medium at 28 °C and treated with or without 8 mM H_2O_2 or 3.5 mM KO_2 for 5 days. (B) Quantitative analysis of the relative growth inhibitory rate in the control (WT, CK1 and CK2) and *Skn7*-silenced strains under oxidative stress tolerance conditions. The growth inhibition rate in each strain was calculated as follows: [diameter (untreated) - diameter (treated)] / diameter (untreated)]. Then, the growth inhibition rate in the WT strains was arbitrarily set to 1, and the growth inhibition rates in all other strains were compared with that in the WT strains. (C) *G. lucidum* strains were grown on a CYM solid agar medium at 28 °C and treated with or without 0.01% SDS or 2 mg mL^{-1} CR for 5 days. (D) Quantitative analysis of the relative inhibitory rate in the control (WT, CK1 and CK2) and *Skn7*-silenced strains during cell wall stress tolerance conditions. The

growth inhibition rate in each strain was calculated as follows: [diameter (untreated) - diameter (treated)] / diameter (untreated). Then, the growth inhibition rate in the WT strains was arbitrarily set to 1, and the growth inhibition rates in all other strains were compared with that in the WT strains. Values are the mean ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the control (WT, CK1 and CK2) strains (**, $P < 0.01$, Student´s t-test).

FIG.6 Cell wall components in *GlSkn7* knockdown strains were decreased.

The control (WT, CK1 and CK2) and *Skn7*-silenced *G. lucidum* strains were cultured on CYM plates at 28 °C for 7 days. (A) *β*-1,3-glucan concentrations in the strains tested. (B) Chitin concentrations in the strains tested. (C) The expression of the putative glucan synthase genes in the strains tested. (D) The expression of the putative chitin synthase genes in the strains tested. The values are the means ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the control (WT, CK1 and CK2) strains (*, $P < 0.05$; **, $P < 0.01$, Student´s t-test).

FIG.7 The thickness of the cell wall in *GlSkn7* knockdown strains was decreased.

(A) The red arrows indicate the inner and outer edges of the cell wall layers. Scale bar = 100 nm. (B) The thickness of the cell wall in all tested strains. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the control (WT, CK1 and CK2) strains (**, $P < 0.01$, Student´s t-test).

FIG.8 Effect of the *GlSkn7* on GA biosynthesis.

(A) Measurement of GAs concentrations in the control (WT, CK1 and CK2) and *Skn7*-silenced strains respectively cultured for 7d. (B)-(D) The transcriptional levels of three key enzymes in GA biosynthesis: hmgr (encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase), sqs (encoding squalene synthase), and osc (encoding lanosterol synthase) in the strains tested. Three independent biological replicates were performed for all experiments. The values are the means ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the control (WT, CK1 and CK2) strains (**, $P < 0.01$, Student´s t-test).

FIG.9 The ROS levels in *G. lucidum* after the ROS scavenger (NAC) treatment.

(A) The control (WT, CK1 and CK2) and *Skn7*-silenced *G. lucidum* strains were cultured at 28 °C on CYM solid agar medium with or without 5 mM NAC for 4 days. The change in the ROS levels was measured by DCFH-DA staining. Scale bar = 100 μm. (B) Changes in the ROS fluorescence ratio in the hyphal regions of the control (WT, CK1 and CK2) and *Skn7*-silenced strains. The y-axis represents the ROS fluorescence ratio as measured by CLSM, and the x-axis represents the different strains. The values in each column represent the captured fluorescence ratio in each observation. (C) Measurement of the amount of H_2O_2 in *G lucidum* with or without the 5 mM NAC treatment. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Different letters

indicate significant differences between the lines ($P < 0.05$ according to Duncan´s multiple range test).

FIG.10 Effect of ROS scavenger (NAC) treatment on GA biosynthesis.

(A) Measurement of the amount of total GAs in the control (WT, CK1 and CK2) and *Skn7*-silenced strains with or without the 5 mM NAC treatment in *G. lucidum*. (B)-(D) Seven-day-old liquid mycelia from the control (WT, CK1 and CK2) and *Skn7*-silenced strains were collected to detect the relative expression of key enzyme genes in the GA biosynthetic pathway in strains treated without or with 5 mM NAC. The values are the means ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$ according to Duncan´s test).

FIG.11 The transcript level of *GlSkn7* and the GAs content of *GlSkn7* knockdown strains in stress conditions.

(A) The transcript level of *GlSkn7* in osmotic stress, heat stress, and fungicide conditions. (B) Measurement of the content of GAs in the control (WT, CK1 and CK2) and Skn7-silenced strains in osmotic stress. (C) Measurement of the GAs concentrations in the control (WT, CK1 and CK2) and Skn7-silenced strains in heat stress. (D) Measurement of the GAs concentrations in the control (WT, CK1 and CK2) and Skn7-silenced strains in fungicide conditions. Three independent biological replicates were performed for all experiments. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the control

(WT, CK1 and CK2) strains (**, P < 0.01, Student´s t-test).

FIG S1 Analysis of putative *GlSkn7* binding sites.

Identification of the consensus binding site present in the promoter regions of target genes.

FIG.S2 Transcript levels of proteins regulated by Yap1 in GlSkn7 knockdown strains.

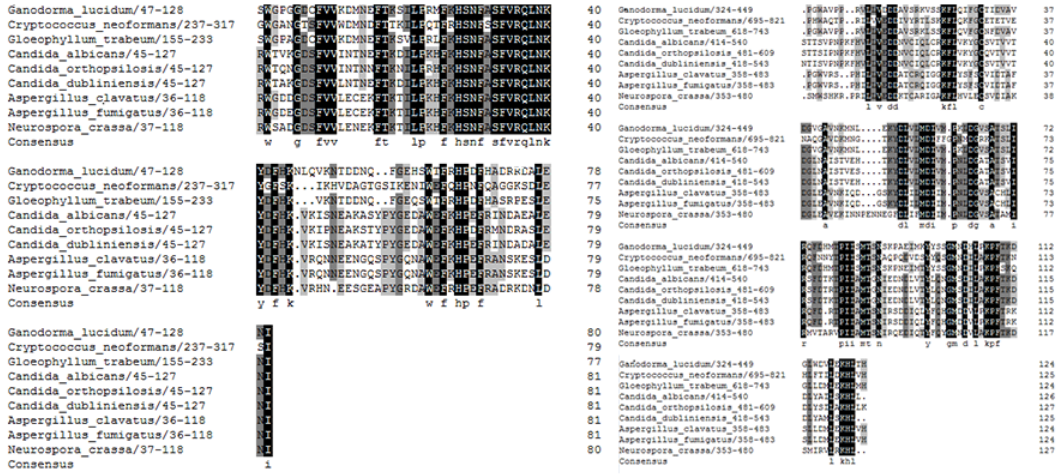
(A) The transcript levels of Yap1-dependent and Skn7-independent genes were measured in the strains tested. (B) The transcript levels of Yap1-dependent and Skn7-dependent genes were measured in the strains tested. Three independent biological replicates were performed for all experiments. The values are the mean ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the control (WT, CK1 and CK2) strains (**, P < 0.01, Student´s t-test).

FIG.S3 Effect of the *GlSkn7* on the GAs biosynthesis along the different phases.

Measurement of the total ganoderic acids concentrations in the control (WT, CK1 and CK2) and Skn7-silenced strains which were cultured respectively for 3d, 4d, 5d, 6d and 7d in liquid CYM medium. Three independent biological replicates were performed for all experiments. The values are the means ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant diff erences from the control (WT, CK1 and CK2) strains (**, P < 0.01, Student´s t-test).

FIG.S4 Transcript levels of genes regulated by Mog-1 in *GlSkn7* knockdown strains.

The transcript levels of genes regulated by Mog-1 were measured in all strains tested. Three independent biological replicates were performed for all experiments. The values are the mean ± standard deviations (SD) of the results of three independent experiments.



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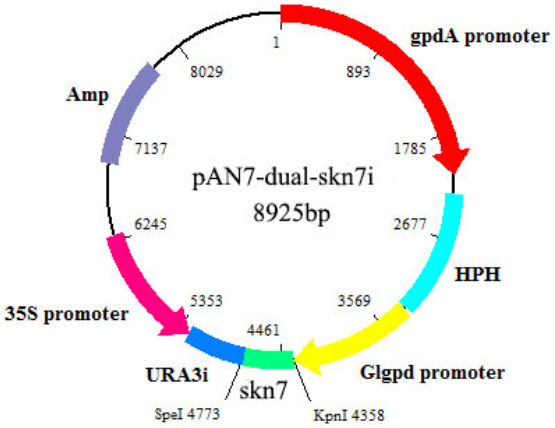

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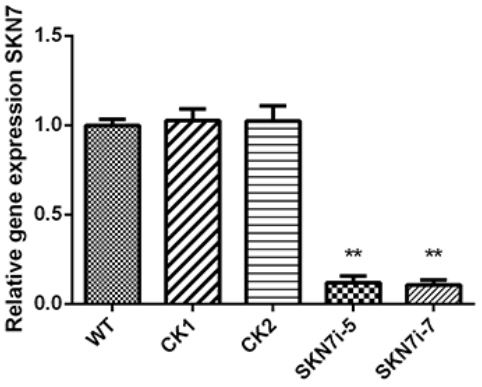

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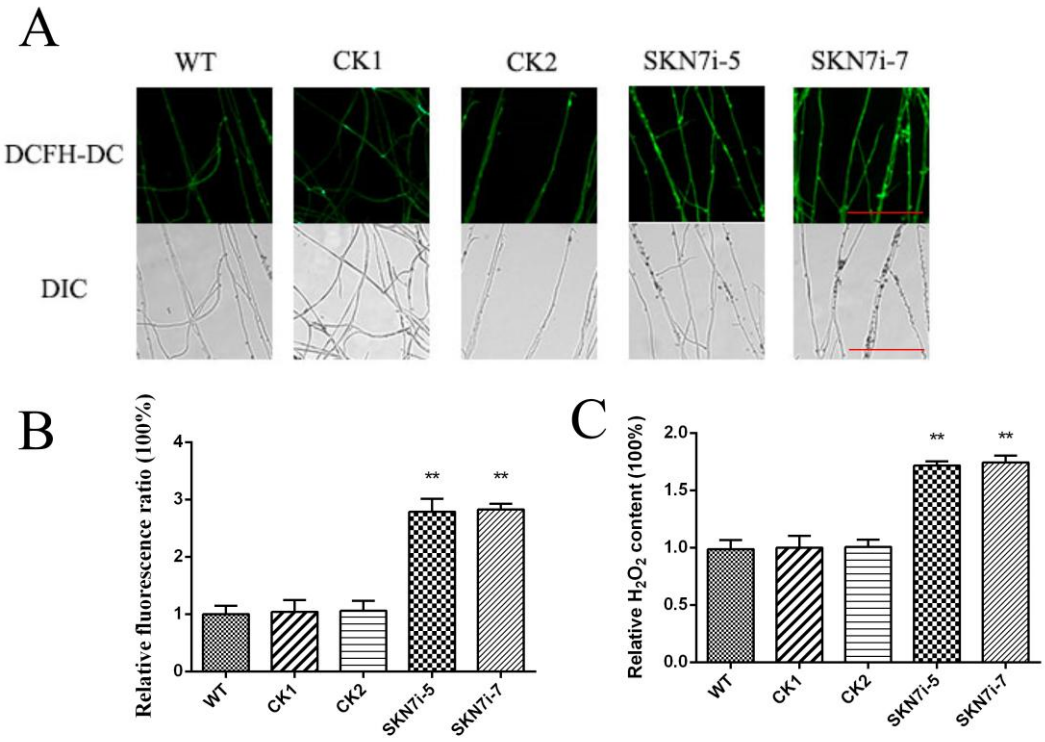

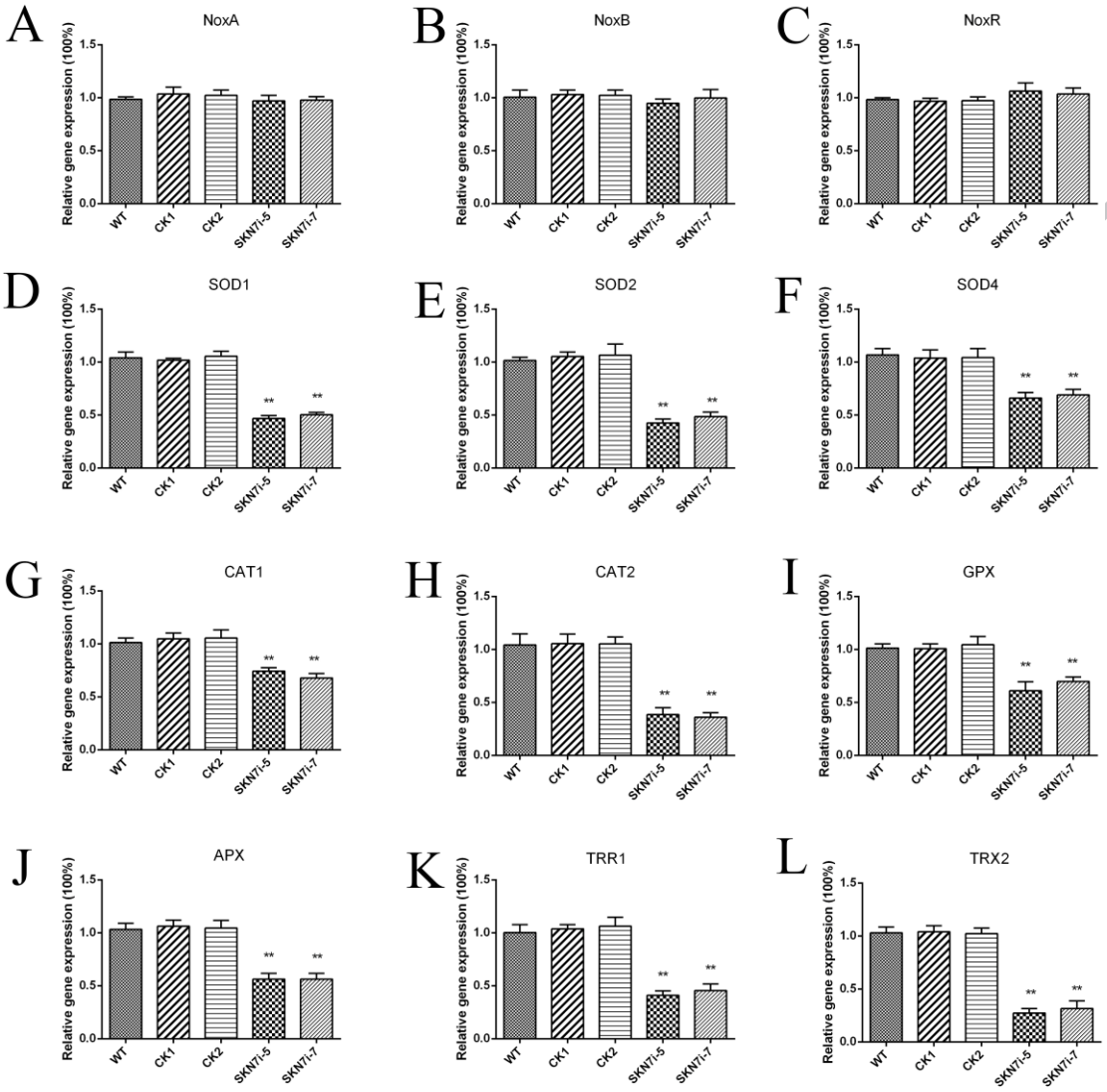

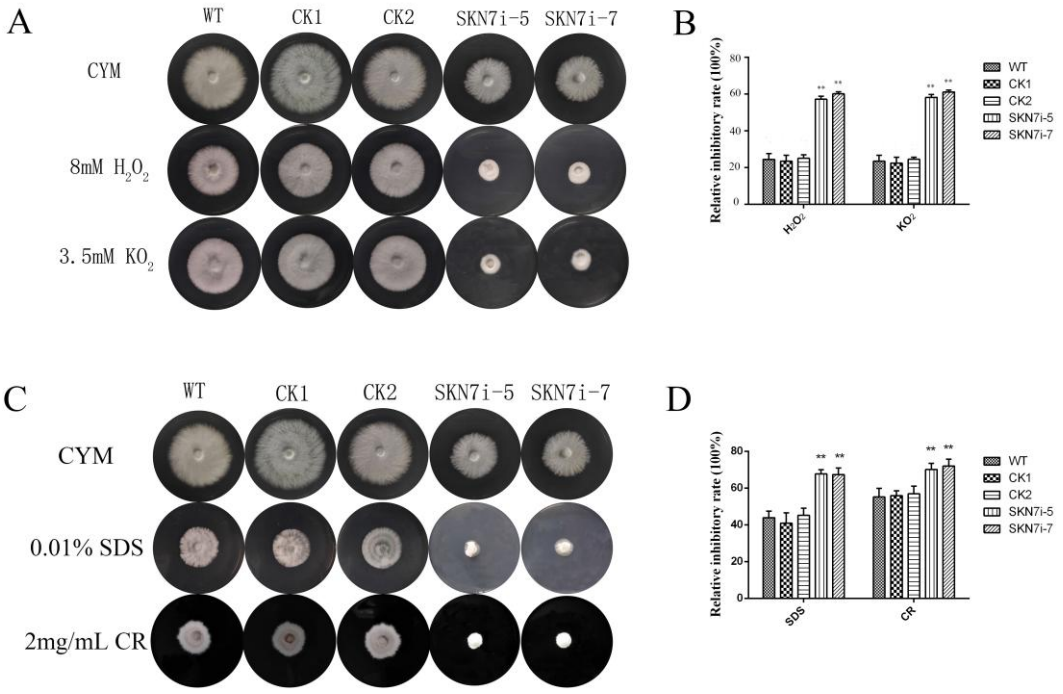

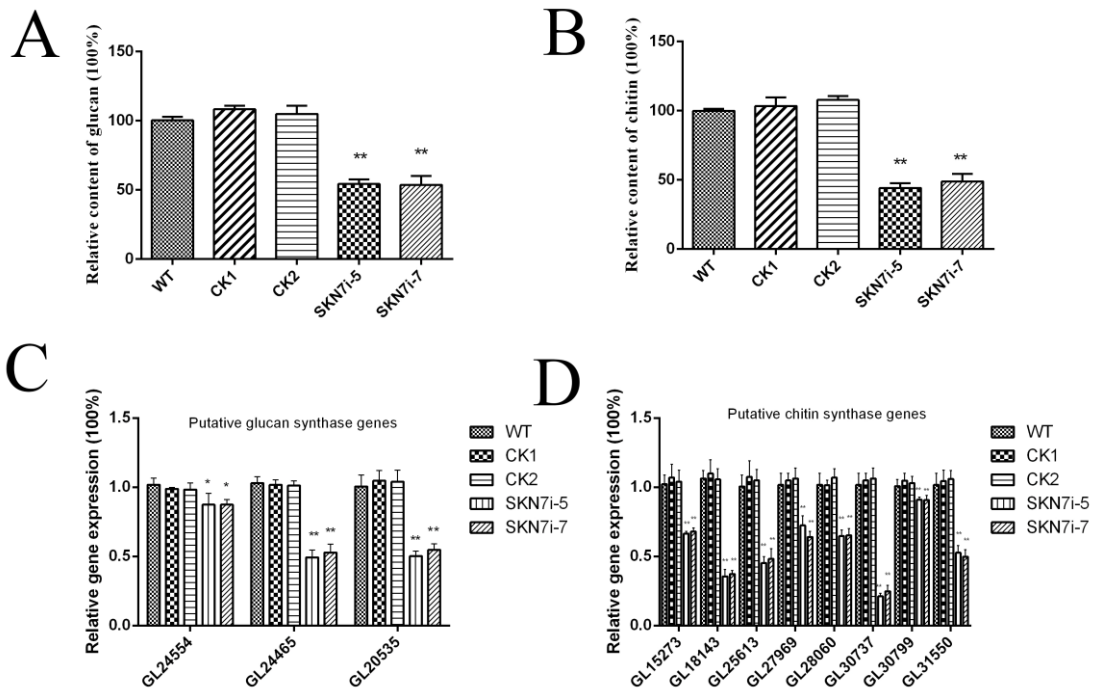

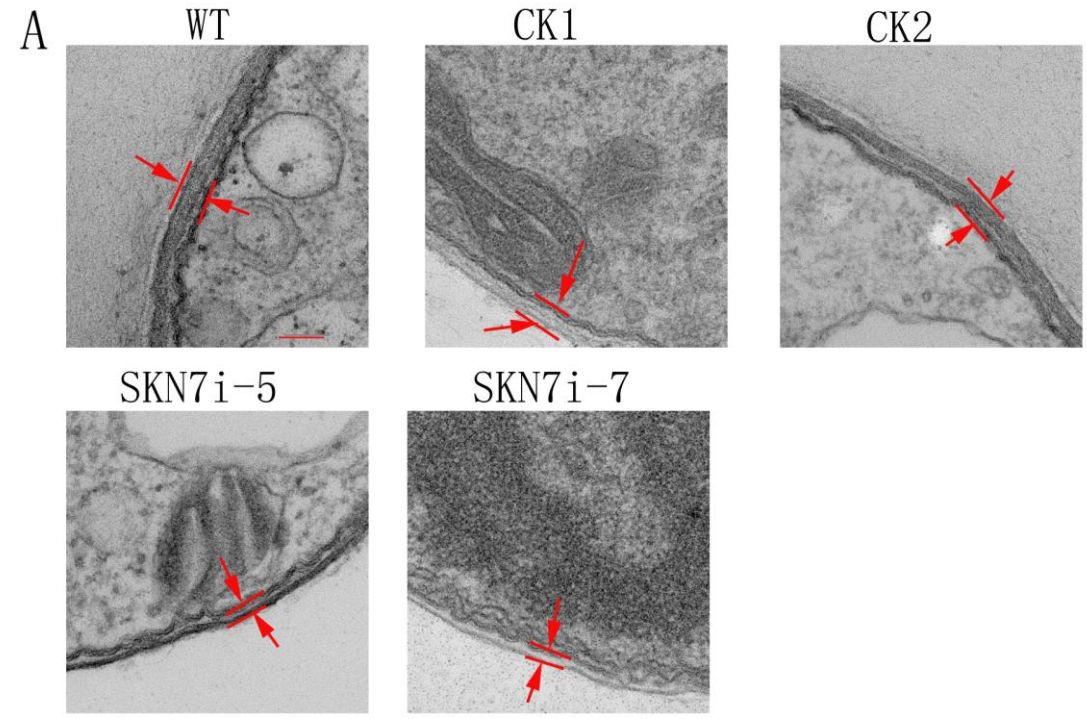

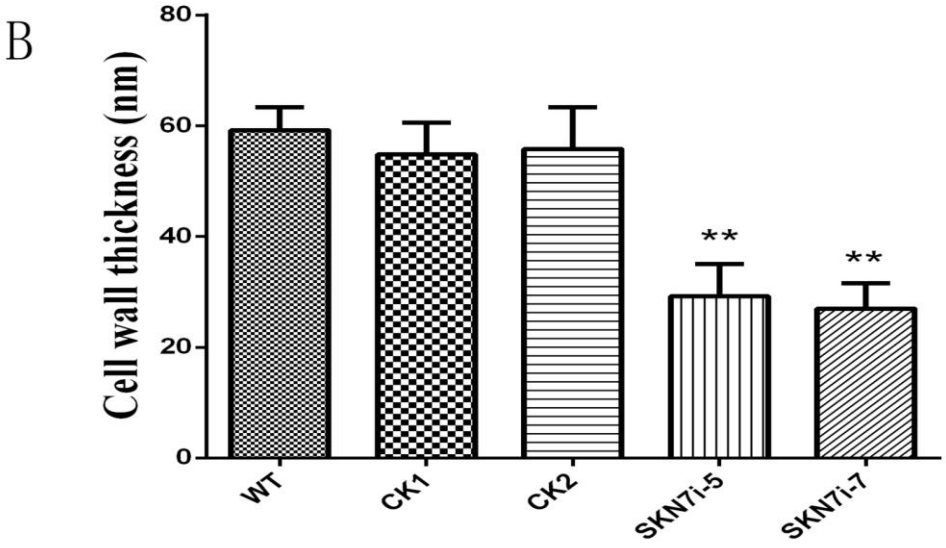

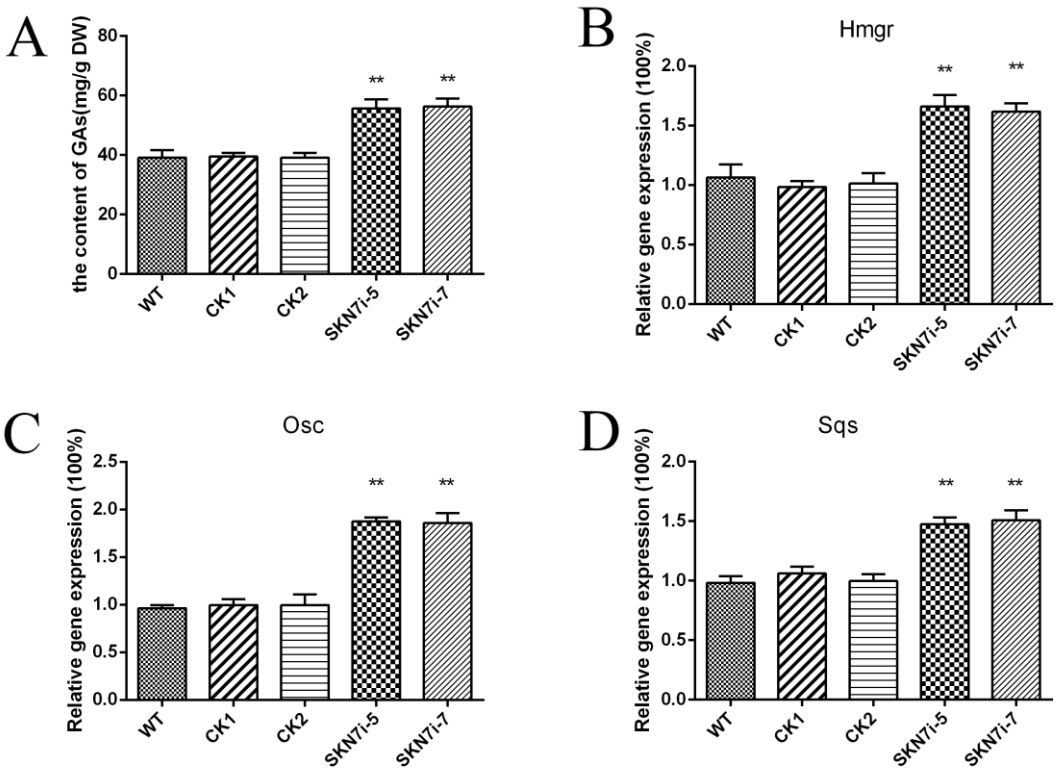

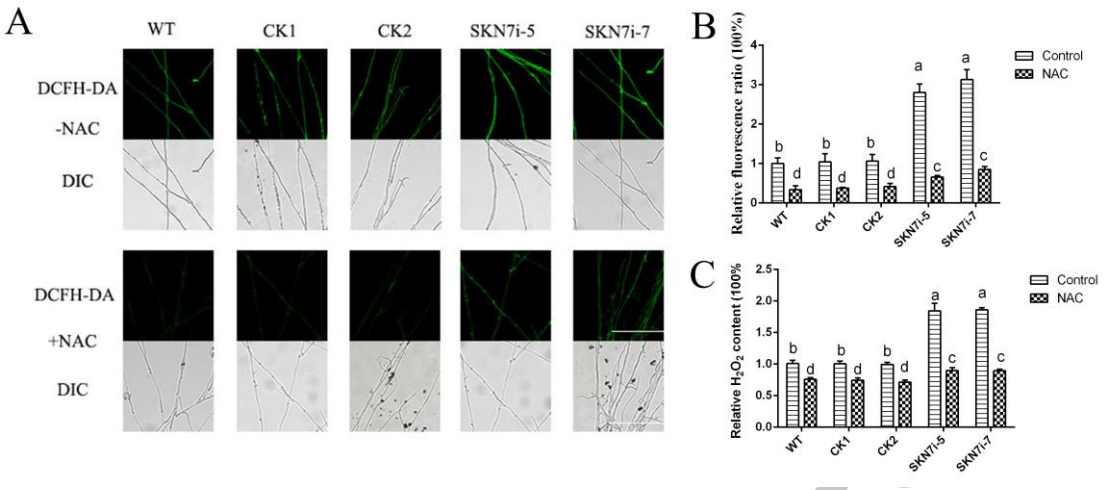

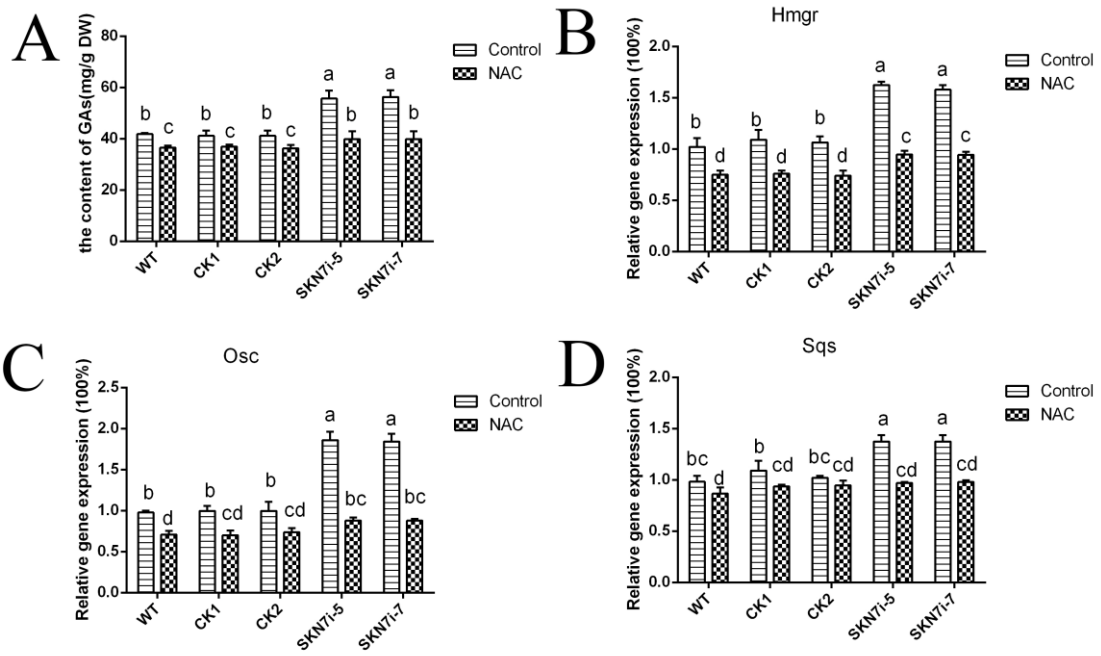

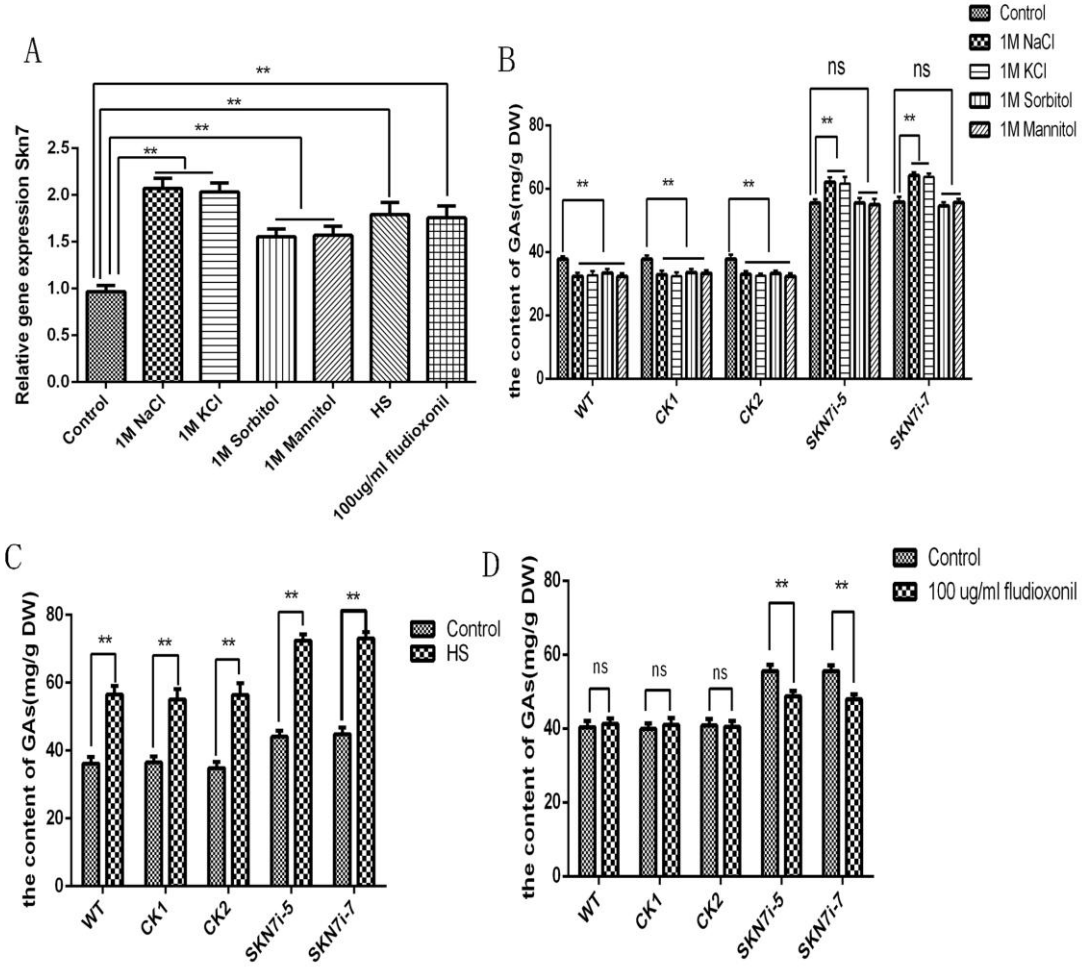

Highlights

- SKN7 regulates intracellular ROS level in *G. lucidum*.
- SKN7 influences cell wall integrity in *G. lucidum*.
- SKN7 influences ganoderic acid biosynthesis of *G. lucidum*.

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