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Functional analysis of an APSES transcription factor (*GlSwi6*) involved in fungal growth, fruiting body development and ganoderic-acid biosynthesis in *Ganoderma lucidum*

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ABSTRACT

The APSES transcription factors have been identified as key regulators of fungal development and other biological processes in fungi. In the present study, the function of *Ganoderma lucidum GlSwi6*, a homolog of *Saccharomyces cerevisiae Swi6*, was characterized. RNAi was used to examine the function of *GlSwi6* in *G. lucidum*. Silencing *GlSwi6* resulted in multiple developmental defects, including reduced fungal growth and increased hyphal branching, and the *GlSwi6*-silenced strains did not exhibit primordium or fruiting body formation. In addition, the H_2O_2 and ganoderic-acid (GA) levels of the *GlSwi6*-silenced strains decreased approximately 50% and 25%, respectively, compared with those of the WT strain. Furthermore, the addition of H_2O_2 led to the recovery of the GA levels of *GlSwi6*-silenced strains, implying that *GlSwi6* might regulate GA biosynthesis by regulating the intracellular ROS levels. Taken together, these results indicate that *GlSwi6* is involved in fungal growth, development and GA biosynthesis in *G. lucidum*.

1. Introduction

Ganoderma lucidum is a wood-degrading basidiomycete with hard fruiting bodies (Boh et al., 2007). In recent years, *G. lucidum* has attracted worldwide attention mainly because of the pharmacologically active secondary metabolites produced by this organism. As therapeutic fungal biofactories, *G. lucidum* were found to produce a wealth of bioactive triterpenoids, polysaccharides, oligosaccharides, peptides, proteins, alcohols and phenols (Shiao, 2003; Shi et al., 2010; Kues et al., 2015). Ganoderic-acid (GA) is believed to be one of the main active components among the various active compounds of this fungus and is often considered to be responsible for the pharmacological effects of this fungus in various human diseases (Sanodiya et al., 2009; Chen et al., 2012). With the sequencing of the *G. lucidum* genome, in recent years, this organism has become a model organism in medicine for the study of secondary metabolic biosynthesis (Chen et al., 2012; Xu et al., 2015). Therefore, investigation of the fundamental biological features of *G. lucidum* is of great importance.

The APSES transcription factor *Swi6* is a target of *Slt2/Mpk1* in *S. cerevisiae* (Levin, 2011) and is involved in the regulation of meiotic initiation (Purnapatre et al., 2002) and G1/S transition of the cell cycle (Coic et al., 2006) as well as of responses to DNA damage (Sidorova and Breeden, 1997). The Swi6 transcription factors from other fungi, such as *MoSwi6* of *Magnaporthe oryzae* (Qi et al., 2012) and *FgSwi6* of *Fusarium graminearum* (Liu et al., 2013), are involved in fungal growth, development and pathogenicity. In addition, the APSES (Asm1, Phd1, Sok1, Efg1, and StuA) family of transcription factors is unique to fungi and is found to regulate gene expression and function in fungal development, morphogenetic processes and other biological processes (Doedt et al., 2004; Ramirez-Zavala and Dominguez, 2008; Zhao et al., 2015). Studies in fungi have indicated that the diverse roles of the APSES transcription factors are differentiated (Zhao et al., 2015). In *M. oryzae*, the *StuA* homolog *Mstu1* is required for the efficient mobilization of conidial reserves during appressorial turgor generation, but *Mstu1* is indispensable for pathogenicity (Nishimura et al., 2009). In *Candida albicans*, *Efg1* functions as a repressor, while *Efh1* functions as an activator in the regulation of fungal morphogenesis and metabolism (Connolly et al., 2013). However, studies of the role of APSES transcription factors in basidiomycetes are relatively rare compared with those in ascomycetes. Elucidating the biological function of APSES transcription factors in *G. lucidum* will be convenient for future studies in basidiomycetes.

In this paper, *GlSwi6*, an APSES transcription factor, was identified in *G. lucidum*. RNA interference was used to examine the function of

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GlSwi6. The results indicate that *GlSwi6* plays an important role in fungal growth and development and in GA biosynthesis. Further analysis indicates that the intracellular ROS level is involved in the GA biosynthesis regulated by *GlSwi6*. Characterization of *GlSwi6* will promote a better understanding of the APSES transcription factors contributing to the biological characteristics of *G. lucidum* and other fungi.

2. Methods and materials

2.1. Fungal strains, culture conditions and dry-weight determination

G. lucidum ACCC53264 from the Agricultural Culture Collection of China was used as the wild-type (WT) strain. The WT, CKs (two independent control strains containing the same empty vector) and *GlSwi6*-silenced strains were cultured on CYM (1% maltose, 2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% $MgSO_4{\cdot}7H_2O$, and 0.46% KH_2PO_4) with or without additional agents for 3–7 days at 28 °C to assess growth and colony characteristics. Fermentation experiments were performed in liquid CYM as previously described (Zhang et al., 2017a). Mycelia were harvested, washed with distilled water and dried at 60 °C to a constant biomass. After inoculation, fruiting bodies were grown at 28 °C on solid culture medium under a light intensity of 300 Lux and in approximately 85% air humidity as previously reported (Mu et al., 2014). *Escherichia coli* (DH5α) was used for plasmid amplification and was grown in lysogeny broth containing ampicillin (100 μg/mL) or kanamycin (50 μg/mL) when needed.

2.2. Cloning and sequence analysis of the GlSwi6 gene

The sequence of *G. lucidum Swi6* was obtained by aligning the amino acid sequence of *S. cerevisiae* Swi6p with the sequence of the *G. lucidum* genome (http://www.herbalgenomics.org/galu/). The entire *GlSwi6* coding sequence was amplified by PCR from *G. lucidum* cDNA using the primers listed in TableS1 and cloned into the pMD19-T vector (TaKaRa, China) for sequencing. The ORF and exon/intron positions were deduced from comparisons of the genomic and cDNA sequences. The theoretical molecular weights of the protein were calculated using the online ExPASy tool (http://expasy.org/tools/pi_tool.html). NCBI Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd) and Pfam (http://smart.embl-heidelberg.de/) were used to identify potential motifs present in GlSwi6. The phylogenetic tree was assembled with ClustalW (Thompson et al., 1994) and MEGA 6.0 (Tamura et al., 2013) and inferred by the neighbor-joining method with 1000 bootstrap replicates.

2.3. Construction of RNAi plasmids and strains

The targeted gene silencing vector pRNAi-Swi6 was constructed using pAN7-dual. Briefly, the sense fragments of *GlSwi6* (466 bp) were prepared by PCR with primers GlSwi6-*Kpn*I-F and GlSwi6-*Spe*I-R, as listed in TableS1, and then digested and inserted into pAN7-dual at the corresponding restriction sites, generating the final construct pRNAi-Swi6 (Fig. S1A). This plasmid was used to transform the *G. lucidum* strain by electroporation (Mu et al., 2012), and all the transformants were first selected on CYM supplemented with 100 μg/mL Hyg.

2.4. Quantitative real-time PCR (qRT-PCR) analysis of gene expression

To analyze the expression of GA-related genes and ROS-related genes, *G. lucidum* was cultured at 28 °C in liquid CYM. To analyze *GlSwi6* expression at different stages, the mycelium was isolated from the three stages of WT *G. lucidum* (mycelium stage, primordium stage and fruiting-body stage) in solid cultures. The levels of the gene-specific messenger RNA (mRNA) expressed by WT and *GlSwi6*-silenced transformants were assessed using qRT-PCR as described in previous studies (Zhang et al., 2017b). The primers for GA-related genes were used as previously reported (Zhang et al., 2017a) and the other primers used are listed in TableS1.

2.5. Determination of hyphal branching

Determination of the lengths between hyphal branches and the statistics of protoplasts generated from the mycelia of WT and *GlSwi6*-silenced strains were conducted as previously described method (Li et al., 2015b).

2.6. Growth susceptibility assay

To test the sensitivity of WT and *GlSwi6*-silenced strains to oxidative stresses, a 6-mm-diameter hyphal tip plug was inoculated onto a CYM plate supplemented with 8 mM H_2O_2 or 8 mM VK_3. The diameters of the strains were determined after incubation at 28 °C for 5–7 days.

2.7. ROS detection assay

DCFH-DA staining (2′,7′-dichlorodihydrofluorescein diacetate) for determination of the ROS content in WT and *GlSwi6*-silenced strains was conducted as previously described (Mu et al., 2014). The H_2O_2 content in WT and *GlSwi6*-silenced strains was determined by using a commercial Hydrogen Peroxide Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) (Zhang et al., 2017b).

2.8. Enzymatic activity assays

The superoxide dismutase (SOD) activity and catalase (CAT) activity were measured as previously described (Zhang et al., 2017b). Total NADPH oxidase (NOX) activity in the hyphae of WT and *GlSwi6*-silenced strains was determined using a previously reported method with slight modification (Zhang et al., 2009). The mycelium was collected from 700 μL of liquid cultures grown in CYM, which was followed by the addition 700 μL of Tris-HCl (50 mM, pH 7.5). Then, XTT (0.5 mM) was added, which was followed by the addition of 50 μM NADPH to activate the reaction. The reaction solution was centrifuged (200*g*, 30 s) to pellet the mycelium after the desired time of treatment. Supernatants were collected for spectrophotometric analysis of XTT formazan production at A_{470}. NOX activity was calculated based on O_2^- production per minute per milligram of mycelium with or without 100 units of SOD.

2.9. Detection and quantification of GA and intermediates

Total GA was extracted from the fungal mycelia and measured by following previously described methods (Ren et al., 2010). Cellular squalene and lanosterol were extracted and measured as described previously (Shi et al., 2015).

2.10. Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.00 for Windows (San Diego California, USA, www.graphpad.com). Averages were calculated for data from at least three independent sample measurements, and the results were expressed as the mean ± standard error (SE). The error bars represent standard deviations from the means of triplicates. The significance of the differences between the analyzed samples was determined by analysis of variance, and sample means were separated by one-way ANOVA and Tukey's honest significant difference test ($P < 0.05$).