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ORIGINAL PAPER

Molecular characterization and expression analysis of *GlHMGS*, a gene encoding hydroxymethylglutaryl-CoA synthase from *Ganoderma lucidum* (Ling-zhi) in ganoderic acid biosynthesis pathway

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Abstract A hydroxymethylglutaryl-CoA synthase gene, designated as *GlHMGS* (GenBank accession No. JN391469) involved in ganoderic acid (GA) biosynthesis pathway was cloned from *Ganoderma lucidum*. The full-length cDNA of *GlHMGS* (GenBank accession No. JN391468) was found to contain an open reading frame of 1,413 bp encoding a polypeptide of 471 amino acid residues. The deduced amino acid sequence of *GlHMGS* shared high homology with other known hydroxymethylglutaryl-CoA synthase (HMGS) enzymes. In addition, functional complementation of *GlHMGS* in a mutant yeast strain *YSC1021* lacking HMGS activity demonstrated that the cloned cDNA encodes a functional HMGS. A 1,561 bp promoter sequence was isolated and its putative regulatory elements and potential specific transcription factor binding sites were analyzed. *GlHMGS* expression profile analysis revealed that salicylic acid, abscisic acid and methyl jasmonate up-regulated *GlHMGS* transcript levels over the control. Further expression analysis revealed that the developmental stage and carbon source had significant effects on *GlHMGS* transcript levels. *GlHMGS* expression peaked on day 16 before decreasing with prolonged culture time. The highest mRNA level was observed when the carbon source was maltose. Overexpression of *GlHMGS* enhanced GA content in *G. lucidum*. This study provides useful information for further studying this gene and on its function in the ganoderic acid biosynthetic pathway in *G. lucidum*.

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Keywords *Ganoderma lucidum* · Hydroxymethylglutaryl-CoA synthase gene · Differential expression · Ganoderic acid biosynthesis · Promoter · Overexpression

Abbreviations

HMGS	Hydroxymethylglutaryl-CoA synthase
GA	Ganoderic acid
ORF	Open reading frame
SA	Salicylic acid
ABA	Abscisic acid
MeJA	Methyl jasmonate
PDA	Potato dextrose agar
PDB	Potato dextrose broth
SEFA PCR	Self-Formed Adaptor PCR
ATMT	*Agrobacterium tumefaciens*-mediated transformation
GPD	Glyceraldehyde-3-phosphate dehydrogenase
HPH	Hygromycin phosphotransferase

Introduction

Ganoderma lucidum (Fr.) Karst, known as Lingzhi in China, is a lamella-less basidiomycetous fungus belonging to the family Polyporaceae. This fungus has been widely used in East Asia as a traditional Chinese medicine where it has a long history of treating a variety of diseases. Pharmaceutical evidences has shown that triterpenoid ganoderic acids are the active components of the fungus that are responsible for anti-androgenic, anti-infammatory, anti-tumor-promoting, and cholesterol synthesis inhibitory activities (Liu et al. 2007; Akihisa et al. 2007; Hajjaj et al. 2005). As secondary metabolite, ganoderic acid (GA) is synthesized via the mevalonate pathway where acetyl-CoA

is converted through a series of chemical reactions to intermediates, and then finally to GA (Shi et al. 2010). Biohydroxymethylglutaryl-CoA synthase (HMGS; E.C. 4.1.3.5) catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to yield HMG-CoA (Chun et al. 2000), a key link between primary metabolism and secondary metabolism, i.e., between the glycolysis pathway and the mevalonate pathway.

HMGS genes and cDNAs were first characterized in the hamster (Gil et al. 1986), and more recently in many other species including *Arabidopsis thaliana* (Montamat et al. 1995), *Brassica juncea* (Alex et al. 2000), human (Aledo et al. 2001) and *Enterococcus faecalis* (Sutherlin et al. 2002). A previous report suggested the presence of two HMG-CoA synthase genes, one that is used in the mitochondrial and the other that functions as a cytosolic enzyme (Ayté et al. 1990). Mitochondrial HMGS, which is only found in vertebrates, catalyzes HMG-CoA to ketones, whereas HMG-CoA, which is produced by cytosolic HMGS, is converted to cholesterol and other isoprenoids via the mevalonate pathway (Servouse and Karst 1986). In the cholesterol biosynthesis pathway, fluvastatin has been shown to increase the transcription of cytosolic *HMGS* by Sterol Regulatory Element (SRE) binding protein (Mascaró et al. 2000). In *B. juncea*, *HMGS* is down-regulated by abscisic acid (ABA), but is up-regulated by methyl jasmonate (MeJA) and salicylic acid (SA). In addition, developmental regulation of HMGS occurs during the flower and seed periods of *B. juncea* (Alex et al. 2000).

In contrast, the molecular characterization of *HMGS* has not been addressed in fungi. In an attempt to elucidate the complete ferrichrome A biosynthetic pathway in *Ustilago maydis*, a cDNA encoding HMGS was fortuitously isolated, and it has been proposed to play a role in HMG-CoA biosynthesis (Winterberg et al. 2010). A tebuconazole-inducible cDNA from *Fusarium graminearum* was shown to encode HMGS, suggesting a role in the resistance of the fungus to the effective fungicide tebuconazole (Liu et al. 2010; Lechoczki-Krsjak et al. 2008). Here, we report the results of the characterization and regulation of *HMGS* in *G. lucidum*. The results show that the developmental stages and carbon sources exhibit significant effects on the *GlHMGS* transcript levels. We also show that SA, ABA and MeJA up-regulate the *GlHMGS* expression.

Materials and methods

Strains, plasmid, and yeast growth conditions

Ganoderma lucidum, strain HG, was obtained from the Shanghai Academy of Agricultural Sciences (Shanghai, China) and was maintained on potato dextrose agar (PDA) medium. The solid culture medium of *G. lucidum* primordia was composed of 78 % cottonseed shell, 20 % wheat bran, 1 % sucrose and 1 % gesso in 60 % to 65 % water. After inoculation, the cultivation bags containing 500 g of solid culture medium were then placed in an incubator at 28 °C for 30 days. Then the primordial was performed at 28 °C with approximately 85 % air humidity and a light intensity of 300 lx. The heterozygous diploid *Saccharomyces cerevisiae* strain YSC1021 (*MAT a/α*, *his3Δ1/his3Δ1*, *leu2Δ0/leu2Δ0*, *lys2Δ0/LYS2*, *Met15/met15Δ0*, *ura3Δ0/ura3Δ0*, *yml126c::kanMX4/YML126c*), lacking the *hmgs* allele (*yml126c*), was purchased from Open Biosystems (Huntsville, AL). Strain YSC1021 was grown in YPD medium (1 % yeast extract, 2 % Bactopeptone, and 2 % glucose) with G418 (200 μg/mL).

RNA and DNA isolation

Mycelia from *G. lucidum* cultures were grown in 250-mL flasks containing 100 mL of potato dextrose broth (PDB). Flasks were shaken on a rotary shaker at 150 rpm at 28 °C for 7 days. Mycelia were harvested, and ground to a fine powder in liquid nitrogen. The total RNA was isolated using an RNA Isolation Kit (Watson, Shanghai, China) according to the manufacturer's recommendations and subsequently treated with DNase I (RNase-free) (Takara). Genomic DNA was isolated from this material by the CTAB method (Saghai-Maroof et al. 1984).

Cloning of the *GlHMGS* gene

Two degenerate primers, HMGS-1 and HMGS-2 (Table 1), were synthesized for amplification of a partial sequence of the genomic DNA (467 bp). The amplification products were ligated into a pMD18-T vector (Takara, Dalian, China), then transformed and sequenced following standard procedures. An attempt was made to obtain a full-length genomic DNA sequence using the Self-Formed Adaptor PCR (SEFA PCR) method (Wang et al. 2007). PCR amplification of the 5′ end of the genomic DNA was first performed using three gene-specific primers (HMGS-Sp1, HMGS-Sp2, and HMGS-Sp3) (Table 1) based upon the 467-bp sequence by SEFA PCR. Total RNA was used to synthesize the first-strand cDNA (3′-ready cDNA) using a 3′-Full RACE Core Set Ver 2.0 Kit (Takara). The 3′-RACE procedure was conducted with HMGS-S and HMGS-In as gene specific outer and inner primers (Table 1). Primers HMGS-3 and HMGS-4 (Table 1), corresponding to the sequences of the 5′- and 3′-termini and incorporating the *BamH*I and *Xba*I restriction sites respectively, were synthesized to obtain the full-length genomic DNA and cDNA. The BLAST program and the

Clustal W program at the US National Center for Biotechnology Information (NCBI) were used for nucleotide sequence analysis, database searches, and amino acid sequence deduction.

Functional complementation of GlHMGS in yeast

pYES2 is a high-copy, autonomously replicating *S. cerevisiae-Escherichia coli* shuttle vector. *GlHMGS* cDNA was ligated into the pYES2 vector (Invitrogen, Carlsbad, CA) downstream of the yeast galactose-dependent GAL1 promoter.

Saccharomyces cerevisiae strain YSC1021 is a diploid yeast. The *HMGS*-deficient haploid yeast ($hmgs^{\Delta}$) was obtained for determining the function of *GlHMGS*, using a series of procedures, as described by Sando et al. (2008). Strain $HMGS^{\Delta}$, which is auxotrophic, was used to investigate the function of the *GlHMGS*. The pYES2 + *GlHMGS* plasmid was used to transform strain $HMGS^{\Delta}$ by the lithium acetate method (Gietz and Schiestl 2007). The diploid *S. cerevisiae* strain YSC1021 was used as wild type. *GlHMGS* transformants were selected on YPG medium.

Software analysis of the *GlHMGS* promoter

The second PCR amplification of the 5′ end of the genomic DNA revealed the promoter sequence. Based on the sequence obtained, primers HMGSp-sp1, HMGSp-sp2, and HMGSp-sp3 (Table 1) were synthesized for amplification of the *GlHMGS* promoter region by SEFA PCR. The promoter sequences were analyzed online using Plantcare software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Expression profiling of *GlHMGS* under various signaling component treatments and at different developmental stages and using different carbon sources in culture

Real-time quantitative RT-PCR (qRT-PCR) was used to investigate the expression profile of *GlHMGS* using primers real-HMGS-1 and real-HMGS-2 (Table 1) (Ren et al. 2010). Under various signaling component treatments, SA, ABA and MeJA were added to PDB to give final concentrations of 100 μM. *G. lucidum* mycelia were harvested after 7 days of growth, with the mycelia treatment with ethanol used as control. To test the expression of *GlHMGS* at different stages in *G. lucidum* development, the fungal mycelia grown in PDB were harvested after 10, 12, 14, 16, 18, and 20 days, respectively. To test the expression of *GlHMGS* under different carbon sources, the original medium (2 % sucrose, 1 % peptone, 0.3 % $KH_2PO_4{\cdot}H_2O$ and 0.15 % $MgSO_4{\cdot}H_2O$) was used. The sucrose was replaced by lactose, mannose, starch, glucose and maltose, respectively. These mycelia were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle to isolate total RNA. The total RNA samples (1 μg) were then reverse-transcribed with M-MLV reverse transcriptase

Table 1 Primers used in this work

Primer	5′→3′	Sequence
HMGS-1	Sense	GARGTSGGNACNGARAC
HMGS-2	Antisense	TCNGGYTTRTARAARTCRTA
HMGS-sp1	Antisense	GG CCGCACGAATGGAAGCGCGGGAAGAAGTG
HMGS-sp2	Antisense	GGAAGAAGTGGGAAAGTGGATGGGATGAATA
HMGS-sp3	Antisense	GGTGGTGGGAACGNNNNNNNNNAGAGCA
HMGS-S	Sense	AATCTACCAAGACCCACCTCATGGACTAC
HMGS-In	Sense	ACTGACGTCGAGGGTGTCGACTCCAA
HMGS-3	Sense	CCAGGATCCATGACCGTCCCCATCAACGCTTC
HMGS-4	Antisense	CTACGATCTAGATCACGCTTTGGGCGCGCGGAT
HMGSp-sp1	Antisense	CCAAGACCGATGGTGTACTTGCCCTTAG
HMGSp-sp2	Antisense	TGGGAAGTACATCTCGATGGCGAGGATAC
HMGSp-sp3	Antisense	AGCCTCGACATCCTGGNNNNNNNNNGCGT
real-HMGS-1	Sense	CCCATCAACGCTTCCACCA
real-HMGS-2	Antisense	GCTCCTCCTCCGAAATGC
real-Gpd-1	Sense	GATGAAGGACTGGCGTGGT
real-Gpd-2	Antisense	CCGTTGAGGCTGGGAATGAC
HPH-4	Sense	TCGTTATGTTTATCGGCACTTT
HPH-5	Antisense	GATGTTGGCGACCTCGTATT
GpdP-F	Sense	CTGGGTATGCGAGGAAGACA

DNA letter codes: H = A, T, or C; Y = C or T; D = A, T, or G; R = G or A; W = A or T; N = A, T, G, or C; S = G or C; B = G, T, or C. The underlined sequences, GGATCC and TCTAGA indicate *Bam* HI and *Xba* I restriction sites respectively

(Takara) using oligo $(dT)_{17}$ as primer, and the synthesized cDNA was used as template for PCR amplification. The relative levels of amplified mRNA were normalized to mRNA expression of the housekeeping gene *G. lucidum* glyceraldehyde-3-phosphate dehydrogenase (*GlGPD*) as an internal control because its expression was found to be stable under our experimental conditions using primers real-Gpd-1 and real-Gpd-2 (Table 1) (Xu et al. 2006; Ren et al. 2010). Gene expression was evaluated by a modification of the $2^{-\triangle\triangle Ct}$ method as described previously (Livak and Schmittgen 2001).

Overexpression analysis of *GlHMGS*

To obtain a better understanding of the role of GlHMGS in GA biosynthesis, we investigated the overexpression of *GlHMGS* in *G. lucidum*. The *GlHMGS* complete cDNA, which was amplified with primers HMGS-3 and HMGS-4 (Table 1), was ligated into pGl-GPG, and the new plasmid was designated as pGL-HMGS. The transformation of *G. lucidum* with pGL-HMGS was conducted using *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Shi et al. 2012). The transformants were selected on CYM medium containing 100 μg/mL hygromycin B. Screening of *GlHMGS* overexpression transformants (OE) was performed by PCR. The detection of the *HPH* gene was conducted using primers HPH-4 and HPH-5 (Table 1). The fusion fragments of the *GlGPD* promoter and the *GlHMGS* gene were detected using primers GpdP-F and HMGS-4.

Measurement of GA content

GA content was determined using the method that has been previously reported (Ren et al. 2010).

Statistical analysis

The data were subjected to ANOVA testing, and the means were assessed for significance by Tukey's test using the SPSS 17.0 package. The results are presented as the mean ± standard deviation.

Results

cDNA cloning and characterization of *GlHMGS*

Based on the highly conserved amino acid regions that are known for HMGS, a 467-bp product was obtained using degenerate primers HMGS-1 and HMGS-2. A BLAST search showed that the PCR product (467 bp) was homologous to a number of known *HMGS* genes. PCR amplification of the 5′ end of the genomic DNA was first performed using three gene-specific primers (HMGS-Sp1, HMGS-Sp2, and HMGS-Sp3) by SEFA PCR (Fig. 1). The full-length DNA was 1, 870 bp and included four introns. The lengths of the introns were 63, 64, 217, and 69 bp, respectively. The full-length cDNA contained a 1, 413 bp ORF, 287 bp of a 3′ noncoding region, and a poly A tail (Fig. 1). The deduced GlHMGS amino acid sequence showed homology with the fungal HMGSs from *Coprinopsis cinerea* (53 % identity, 81 % positive), *Puccinia graminis* (56 % identity, 71 % positive), and *Aspergillus oryzae* (53 % identity, 70 % positive). Sequence analysis has shown that the eukaryotic HMGSs have well-conserved amino acid residues (Glu-100, Tyr-135, His-269, Asn-348 and Ser-382) that are crucial for the activity of HMG-CoA synthase (Sando et al. 2008; Campobasso et al. 2004), and GlHMGS had all five of these conserved sites (Fig. 1).

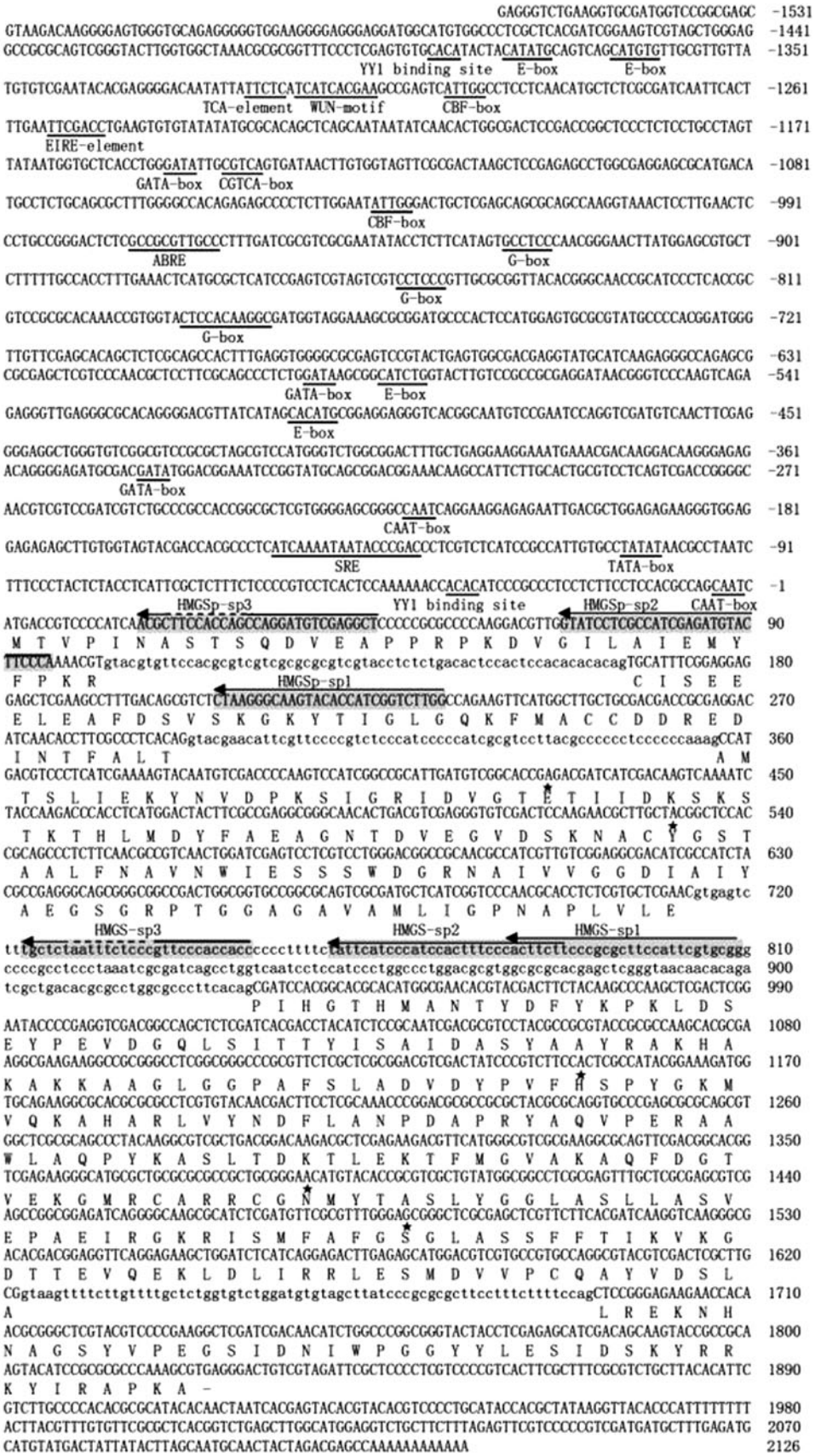
Fig. 1 The promoter region and genomic DNA sequence of *GlHMGS* ▶ from *G. lucidum* and its deduced amino acid sequence. The deduced cis-acting elements with significant similarity to previously identified cis-acting elements are *underlined*, and the name is given under each element. The translational start site (the framed ATG) was defined as position +1. The coded amino acid sequence is shown below the cDNA sequence. The initiation (ATG) and termination codons (TGA) are framed. The positions of SEFA PCR primers are shadowed. The positions of the degenerate primers are *underlined*. The genomic DNA sequence and cDNA sequence of *GlHMGS* was submitted to GenBank (JN391469 and JN391468)

Functional complementation of GlHMGS in yeast

To determine the function of *GlHMGS*, the *hmgs*-deficient haploid yeast $hmgs^{\Delta}$ was obtained for transformation. The $hmgs^{\Delta}$ yeast is an auxotrophic strain. The *GlHMGS* cDNA was ligated to pYES2 downstream of the yeast GAL1 promoter, which is a galactose-dependent promoter. After transformation with vector pYES2 + *GlHMGS*, the $hmgs^{\Delta}$ (pYES2 + *GlHMGS*) transformants grew readily on an induction medium YPG (1 % yeast extract, 2 % Bactopeptone, and 2 % galactose) with G418, whereas they did not grow on a non-induction medium (YPD with G418). The expression of *GlHMGS* can repair the functional defect of the $hmgs^{\Delta}$ yeast. These data indicate that GlHMGS have HMGS activity (Fig. 2).

Sequence features of the *GlHMGS* promoter region

A 1, 561 bp promoter was obtained using primers HMGSp-sp1, HMGSp-sp2, and HMGSp-sp3 (Fig. 1) by SEFA PCR. Three predicted CAAT boxes and a TATA box were found. Several putative regulatory elements associated with hormones, light, elicitor, and stress-related responses were also found in the promoter region, including the CGTCA-motif (MeJA-responsiveness element), ABRE (ABA-responsiveness element), TCA-element (SA-responsiveness element),


GAGGGTCTGAAGGTGCGATGGTCCGGCGAGC -1531
GTAAGACAAGGGGAGTGGGTGCAGAGGGGGTGGAAGGGGAGGGAGGATGGCATGTGGCCCTCGCTCACGATCGGAAGTCGTAGCTGGGAG -1441
GCCGCGCAGTCGGGTACTTGGTGGCTAAACGCGCGGTTTCCCTCGAGTGTGCACATACTACATATGCAGTCAGCATGTGTTGCGTTGTTA -1351
YY1 binding site E-box E-box
TGTGTCGAATACACGAGGGGACAATATTATTCTCATCATCACGAAGCCGAGTCATTGGCCTCCTCAACATGCTCTCGCGATCAATTCACT -1261
TCA-element WUN-motif CBF-box
TTGAATTCGACCTGAAGTGTGTATATATGCGCACAGCTCAGCAATAATATCAACACTGGCGACTCCGACCGGCTCCCTCTCCTGCCTAGT -1171
EIRE-element
TATAATGGTGCTCACCTGGGATATTGCGTCAGTGATAACTTGTGGTAGTTCGCGACTAAGCTCCGAGAGCCTGGCGAGGAGCGCATGACA -1081
GATA-box CGTCA-box
TGCCTCTGCAGCGCTTTGGGGCCACAGAGAGCCCCTCTTGGAATATTGGGACTGCTCGAGCAGCGCAGCCAAGGTAAACTCCTTGAACTC -991
CBF-box
CCTGCCGGGACTCTCGCCGCGTTGCCCTTTGATCGCGTCGCGAATATACCTCTTCATAGTGCCTCCCAACGGGAACTTATGGAGCGTGCT -901
ABRE G-box
CTTTTTGCCACCTTTGAAACTCATGCGCTCATCCGAGTCGTAGTCGTCCTCCCGTTGCGCGGTTACACGGGCAACCGCATCCCTCACCGC -811
G-box
GTCCGCGCACAAACCGTGGTACTCCACAAGGCGATGGTAGGAAAGCGCGGATGCCCACTCCATGGAGTGCGCGTATGCCCCACGGATGGG -721
G-box
TTGTTCGAGCACAGCTCTCGCAGCCACTTTGAGGTGGGGCGCGAGTCCGTACTGAGTGGCGACGAGGTATGCATCAAGAGGGCCAGAGCG -631
CGCGAGCTCGTCCCAACGCTCCTTCGCAGCCCTCTGGATAAGCGGCATCTGGTACTTGTCCGCCGCGAGGATAACGGGTCCCAAGTCAGA -541
GATA-box E-box
GAGGGTTGAGGGCGCACAGGGGACGTTATCATAGCACATGCGGAGGAGGGTCACGGCAATGTCCGAATCCAGGTCGATGTCAACTTCGAG -451
E-box
GGGAGGCTGGGTGTCGGCGTCCGCGCTAGCGTCCATGGGTCTGGCGGACTTTGCTGAGGAAGGAAATGAAACGACAAGGACAAGGGAGAG -361
ACAGGGGAGATGCGACGATATGGACGGAAATCCGGTATGCAGCGGACGGAAACAAGCCATTCTTGCACTGCGTCCTCAGTCGACCGGGGC -271
GATA-box
AACGTCGTCCGATCGTCTGCCCGCCACCGGCGCTCGTGGGGAGCGGGCCAATCAGGAAGGAGAGAATTGACGCTGGAGAGAAGGGTGGAG -181
CAAT-box
GAGAGAGCTTGTGGTAGTACGACCACGCCCTCATCAAAATAATACCCGACCCTCGTCTCATCCGCCATTGTGCCTATATAACGCCTAATC -91
SRE TATA-box
TTTCCCTACTCTACCTCATTCGCTCTTTCTCCCCGTCCTCACTCCAAAAAACCACACATCCCGCCCTCCTCTTCCTCCACGCCAGCAATC -1
HMGSp-sp3 YY1 binding site HMGSp-sp2 CAAT-box
ATGACCGTCCCCATCAACGCTTCCACCAGCCAGGATGTCGAGGCTCCCCCGCGCCCCAAGGACGTTGGTATCCTCGCCATCGAGATGTAC 90
1 M T V P I N A S T S Q D V E A P P R P K D V G I L A I E M Y
TTCCCAAAACGTgtacgtgttccacgcgtcgtcgcgcgcgtcgtacctctctgacactccactccacacacacagTGCATTTCGGAGGAG 180
31 F P K R HMGSp-sp1 C I S E E
GAGCTCGAAGCCTTTGACAGCGTCTCTAAGGGCAAGTACACCATCGGTCTTGGCCAGAAGTTCATGGCTTGCTGCGACGACCGCGAGGAC 270
40 E L E A F D S V S K G K Y T I G L G Q K F M A C C D D R E D
ATCAACACCTTCGCCCTCACAGgtacgaacattcgttccccgtctcccatcccccatcgcgtccttacgccccctcccccccaaagCCAT 360
70 I N T F A L T A M
GACGTCCCTCATCGAAAAGTACAATGTCGACCCCAAGTCCATCGGCCGCATTGATGTCGGCACCGAGACGATCATCGACAAGTCAAAATC 450
79 T S L I E K Y N V D P K S I G R I D V G T E T I I D K S K S
TACCAAGACCCACCTCATGGACTACTTCGCCGAGGCGGGCAACACTGACGTCGAGGGTGTCGACTCCAAGAACGCTTGCTACGGCTCCAC 540
109 T K T H L M D Y F A E A G N T D V E G V D S K N A C Y G S T
CGCAGCCCTCTTCAACGCCGTCAACTGGATCGAGTCCTCGTCCTGGGACGGCCGCAACGCCATCGTTGTCGGAGGCGACATCGCCATCTA 630
139 A A L F N A V N W I E S S S W D G R N A I V V G G D I A I Y
CGCCGAGGGCAGCGGGCGGCCGACTGGCGGTGCCGGCGCAGTCGCGATGCTCATCGGTCCCAACGCACCTCTCGTGCTCGAACgtgagtc 720
169 A E G S G R P T G G A G A V A M L I G P N A P L V L E
HMGS-sp3 HMGS-sp2 HMGS-sp1
tttgctctaatttctcccgttcccaccaccccctttctattcatcccatccactttcccactttcttcccgcgcttccattcgtgcggg 810
ccccgcctccctaaatcgcgatcagcctggtcaatcctccatccctggccctggacgcgtggcgcgcacgagctcgggtaacaacacaga 900
tcgctgacacgcgcctggcgcccttcacagCGATCCACGGCACGCACATGGCGAACACGTACGACTTCTACAAGCCCAAGCTCGACTCGG 990
196 P I H G T H M A N T Y D F Y K P K L D S
AATACCCCGAGGTCGACGGCCAGCTCTCGATCACGACCTACATCTCCGCAATCGACGCGTCCTACGCCGCGTACCGCGCCAAGCACGCGA 1080
216 E Y P E V D G Q L S I T T Y I S A I D A S Y A A Y R A K H A
AGGCGAAGAAGGCCGCGGGCCTCGGCGGGCCCGCGTTCTCGCTCGCGGACGTCGACTATCCCGTCTTCCACTCGCCATACGGAAAGATGG 1170
246 K A K K A A G L G G P A F S L A D V D Y P V F H S P Y G K M
TGCAGAAGGCGCACGCGCGCCTCGTGTACAACGACTTCCTCGCAAACCCGGACGCGCCGCGCTACGCGCAGGTGCCCGAGCGCGCAGCGT 1260
276 V Q K A H A R L V Y N D F L A N P D A P R Y A Q V P E R A A
GGCTCGCGCAGCCCTACAAGGCGTCGCTGACGGACAAGACGCTCGAGAAGACGTTCATGGGCGTCGCGAAGGCGCAGTTCGACGGCACGG 1350
306 W L A Q P Y K A S L T D K T L E K T F M G V A K A Q F D G T
TCGAGAAGGGCATGCGCTGCGCGCGCCGCTGCGGGAACATGTACACCGCGTCGCTGTATGGCGGCCTCGCGAGTTTGCTCGCGAGCGTCG 1440
336 V E K G M R C A R R C G N M Y T A S L Y G G L A S L L A S V
AGCCGGCGGAGATCAGGGCAAGCGCATCTCGATGTTCGCGTTTGGGAGCGGGCTCGCGAGCTCGTTCTTCACGATCAAGGTCAAGGGCG 1530
366 E P A E I R G K R I S M F A F G S G L A S S F F T I K V K G
ACACGACGGAGGTTCAGGAGAAGCTGGATCTCATCAGGAGACTTGAGAGCATGGACGTCGTGCCGTGCCAGGCGTACGTCGACTCGCTTG 1620
396 D T T E V Q E K L D L I R R L E S M D V V P C Q A Y V D S L
CGgtaagttttcttgtttgctctggtgtctggatgtgtagcttatcccgcgcgcttcctttctttttccagCTCCGGGAGAAGAACCACA 1710
426 A L R E K N H
ACGCGGGCTCGTACGTCCCCGAAGGCTCGATCGACAACATCTGGCCCGGCGGGTACTACCTCGAGAGCATCGACAGCAAGTACCGCCGCA 1800
433 N A G S Y V P E G S I D N I W P G G Y Y L E S I D S K Y R R
AGTACATCCGCGCGCCCAAAGCGTGAGGGACTGTCGTAGATTCGCTCCCCTCGTCCCCGTCACTTCGCTTTCGCGTCTGCTTACACATTC 1890
463 K Y I R A P K A -
GTCTTGCCCCACACGCGCATACACAACTAATCACGAGTACACGTACACGTCCCCTGCATACCACGCTATAAGGTTACACCCATTTTTTTT 1980
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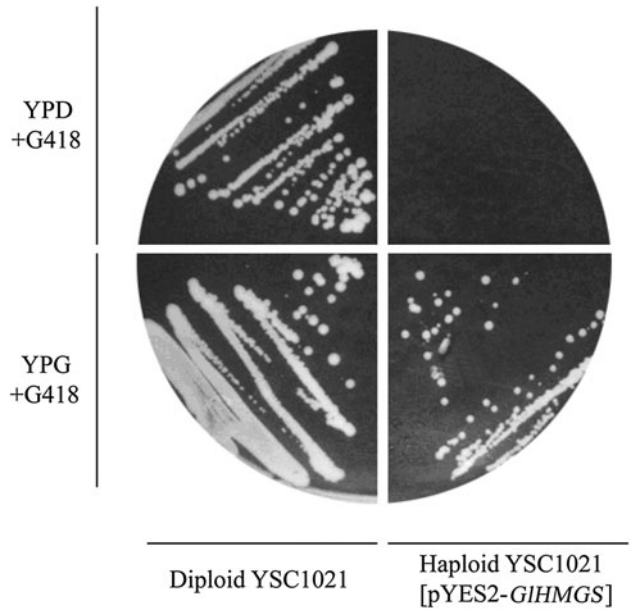

Fig. 2 Functional complementation of *GlHMGS* in mutant yeast strain YSC1021 $hmgs^{\Delta}$. Diploid YSC1021 and haploid YSC1021 [pYES2-GlHMGS] were streaked onto YPD and YPG plates, and incubated at 30 °C for 3 days

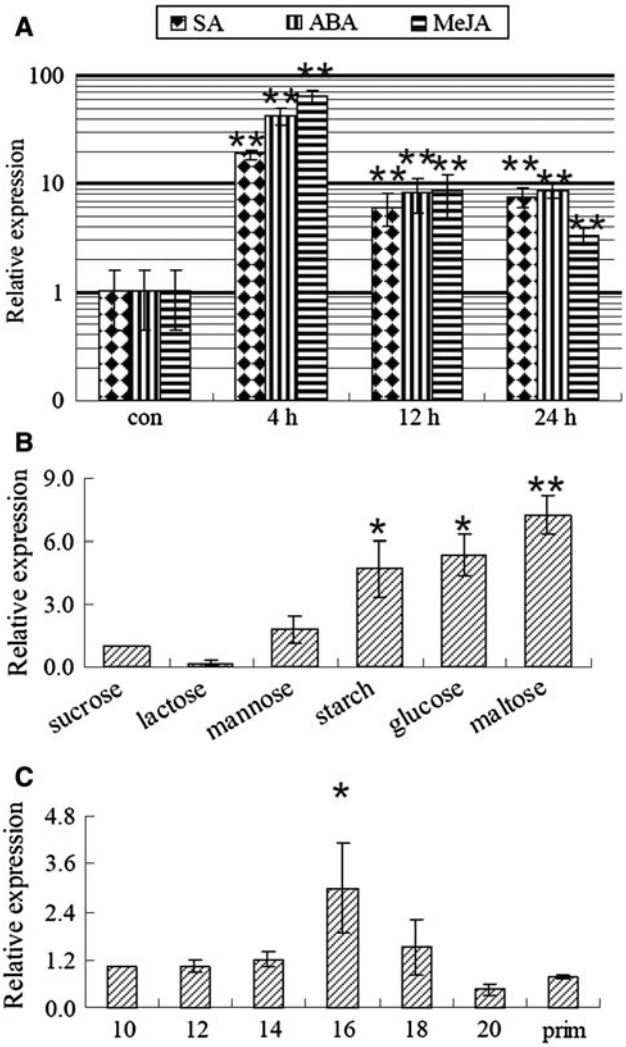

Fig. 3 Expression analysis of the *GlHMGS* gene. **a** The mycelia were treated with signal molecules (SA, ABA, and MeJA). The level of transcript is expressed as the ratio relative to the control CK (treated with ethanol). The qRT-PCR analysis was performed using total RNA isolated from the mycelia. **b** The transcript levels of *GlHMGS* were detected by qRT-PCR under different carbon sources of cultural conditions. The level of transcription is expressed as the ratio relative to the sucrose cultural mycelia. **c** The qRT-PCR analysis of *GlHMGS* transcription in mycelia aged between 10 and 20 days as well as in primordia. Numbers 10, 12, 14, 16, 18, and 20 represent 10, 12, 14, 16, 18, and 20 days old mycelia, respectively. Standard deviations of the means ($N = 3$) are shown as *error bars*. Significant differences are indicated with an *asterisk* at $P < 0.05$ and with *two asterisks* at $P < 0.01$

the Ying Yang 1 (YY1) binding site, a G-box (light-responsiveness element), an E-box (5′-CANNTG-3′), a carbon binding site (5′-SYGGRG-3′), a SRE (sterol regulatory element), a GATA-motif (nitrogen-responsiveness element), a WUN-motif (wound-responsiveness element) and an EIRE-element (elicitor-responsiveness element). Analysis of these specific motifs of the *GlHMGS* promoter region might identify useful cis-acting elements and thus provide some clues to the mechanism by which this gene responds to environmental signals.

Expression profiling of *GlHMGS*

To understand the expression characterization of *GlHMGS*, we treated *G. lucidum* mycelia with the signal molecules, SA, ABA and MeJA and measured mRNA levels by qRT-PCR analysis. Figure 3a shows the effects of SA, ABA and MeJA on *GlHMGS* mRNA levels. *GlHMGS* expression was induced by SA, ABA and MeJA, which peaked at 4 h post-treatment (about 18.81, 41.93, 64.75 folds as compared with control, respectively). When 100 μM SA was added to PDB medium, the GA content reached 2.74 ± 0.25 mg/100 mg DW, which was 1.30 fold compared with the control group (2.11 ± 0.08 mg/100 mg DW). It has also been reported that MeJA induced the GA content. The GA content was 3.72 ± 0.17 mg/100 mg DW under 100 μM MeJA treatment (Ren et al. 2010). The results suggest that the expression of *GlHMGS* might be involved in the processes regulated by SA, ABA and MeJA in *G. lucidum*.

QRT-PCR analysis of *GlHMGS* transcription was performed to determine expression differences caused by different carbon sources in the cultural media (Fig. 3b). The results suggest that *GlHMGS* transcription was regulated by different carbon sources. The highest mRNA level

was observed with maltose as the carbon source (approximate 7.25-fold compared with the sucrose as carbon source). In our previous work, the GA content was improved by maltose (Li et al. 2006). Maltose utilization and regulation in fungi is of great importance for academic and industrial research (Vongsangnak et al. 2009). These results further suggest that metabolism and gene expression may be regulated by maltose in *G. lucidum*. Figure 3c describes *GlHMGS* transcription in mycelia that were between 10 and 20 days old and in primordia. Gene expression peaked on day 16 (approximate 2.98-fold compared with 10 days-old mycelia). The changing rule of *HMGS* gene expression is consistent with the findings of Alex et al. who observed that developmental regulation occurs in the flower and seed of *B. juncea* (Alex et al. 2000).

Overexpression of *GlHMGS*

G. lucidum wild type strain was transformed with pGL-HMGS. The transformants were selected on CYM medium containing 100 μg/mL hygromycin B. The randomly choose transformants were detected using a PCR analysis to determine whether HPH fragment and fusion fragment were integrated into the genomic DNA (Fig. 4a, b). We randomly choose OE-1, OE-6, and OE-7 to check the transcript levels of *GlHMGS*. The data showed that the *GlHMGS* was overexpressed in three OEs (Fig. 4c). The transcript level of *GlHMGS* of OE7 was 2.3-fold compared to the wt. The GA content also was detected in the three OEs. The results showed that GA content was approximately 15.1–24.2 % higher than wt group (Fig. 4d). These results further revealed that *GlHMGS* involved in the GA biosynthesis pathway.

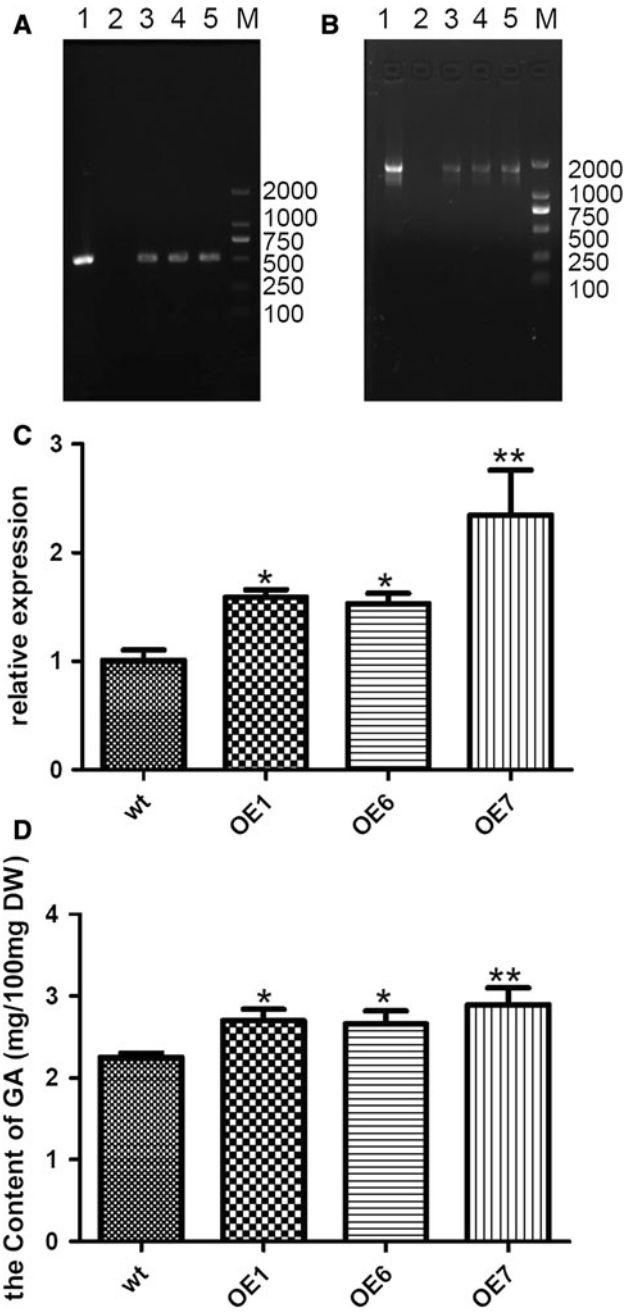

Fig. 4 Identification and characterization of the OEs. **a** Amplification of hph gene fragment in genomic DNA isolated from OEs. *Lane 1* pGl-HMGS as positive control, *Lane 2* negative control with untransformed *G. lucidum* wild-type (wt), *Lanes 3–5* OE-1, OE6, and OE7, *Lane M* DL 2000 DNA Marker. **b** Amplification of fusion fragment *GlGPD* promoter-*GlHMGS* gene in genomic DNA isolated from OEs. *Lane 1* pGl-HMGS as positive control, *Lane 2* negative control with wt, *Lanes 3–5*: OE-1, OE6, and OE7, *Lane M* DL 2000 DNA Marker. **c** The transcript levels of the *GlHMGS* in wt and OEs. **d** The GA content in wt and OEs

Discussion

HMGS has recently been shown to catalyze the first step in the mevalonate biosynthesis pathway (Bahnson 2004). HMGSs share a high sequence identity with strict conservation of all of the residues in the active sites located at the active site entrance (Pojer et al. 2006). Five amino acid residues (Glu-100, Tyr-135, His-269, Asn-348, and Ser-382) crucial for the catalytic activity have also been found in GlHMGS (Fig. 1). These results suggest that the active sites of all HMGS are highly conserved.

The regulation of secondary metabolic biosynthesis in fungi is often a complex regulation network, involving growth, differentiation, and development (Calvo et al. 2002; Xu et al. 2011). In *B. juncea*, developmental regulation of *BjHMGS* occurs in the flower and seed (Alex et al. 2000). The up-regulation of *GlHMGS* was detected at the middle stage of the mycelia (Fig. 3c). Our previous study indicated that the GA content (3.80 mg/100 mg DW) at the primordia stage was 1.55 fold compared with the mycelia

stage (2.45 mg/100 mg DW) (Zhao et al. 2004). The gene expression is not complete consistency with GA production under different developmental stage. The possible reason is that the gene HMGS is located in the upstream of the GA biosynthesis pathway. The increase of GA content is the result of biosynthesis and accumulation. The *GlHMGS* expression may be earlier than the increase of GA content. As secondary metabolite, GA biosynthesis in *G. lucidum* was affected by a variety factors (Shi et al. 2010), except that during the developmental stage. The promoter of *GlHMGS* was isolated and characterized, and we found various environmental response elements in the promoter of *GlHMGS* (Fig. 1). It has been inferred that gene expression of *HMGS* is regulated by environmental factors via various response elements. SA, ABA and MeJA have been implicated as abiotic elicitors with multible effects on plant growth, development, and secondary metabolism (Georgiev et al. 2007). Exogenously applied MeJA stimulated GA production in cultured *G. lucidum* mycelia (Ren et al. 2010). In fact, *GlHMGS* expression was induced by SA, ABA and MeJA (Fig. 3a). A further interesting work is whether *GlHMGS* dependent on those putative response elements involved in the regulation of the GA biosynthesis pathways of signal molecular.

As the major pharmacologically active constituents of *G. lucidum*, GA is synthesized via the mevalonate pathway. The first step in this pathway involves the catalysis of acetyl-CoA to HMG-CoA by HMGS. Acetyl-CoA is the central intermediate in carbon metabolism (Carman et al. 2008). Its metabolism involves a complex multibranched metabolic network (Sørensen et al. 2009). Therefore, *HMGS* plays an important role in the control of metabolic flux in the mevalonate pathway (Wang et al. 2012). Changes in the transcription of *GlHMGS* that were dependent on carbon source indicated that GlHMGS may mediate adaptation to various carbon sources in *G. lucidum* (Fig. 3b). In previous work reported by our group, the GA content were 2.43 ± 0.04, 1.37 ± 0.03, 1.24 ± 0.03, 1.23 ± 0.01, 1.04 ± 0.02, and 0.97 ± 0.02 mg/100 mg DW when the carbon source was sucrose, glucose, maltose, mannose, lactose, and starch, respectively (Li et al. 2006). Acetyl-CoA has been shown to affect transcription in vitro (Galasinski et al. 2000). As the substrate of GlHMGS enzyme, acetyl-CoA represents an important substrate for regulating metabolic activity, which provides metabolic feedback into the regulation of gene expression. These observations suggest that *GlHMGS* probably plays an important role in the metabolic flux control during GA biosynthesis by *G. lucidum*.

In conclusion, for the first time, we have successfully isolated and characterized the full-length cDNA and the promoter region of *GlHMGS* from the medicinal fungus, *G. lucidum*. The *GlHMGS* was shown to complement yeast mutants defective in HMGS. The expression of *GlHMGS* changed rapidly in response to signal molecules (SA, ABA, and MeJA), which indicated that it was inducible and might be involved in signal molecule response to environmental stimuli. In addition, the *GlHMGS* transcription was also induced more readily by maltose than sucrose. The overexpression of *GlHMGS* enhanced the GA biosynthesis in *G. lucidum*. The present study should be helpful for investigations aimed at understanding the detailed role of *GlHMGS* in GA biosynthesis by *G. lucidum* at the molecular genetic level.

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