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In silico Analysis of three Committed-Steps Involved in Triterpenoid Biosynthesis from *Ganoderma lucidum*

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Abstract: *Ganoderma lucidum* is widely considered as one of the most valuable Traditional Chinese Medicines, with various bioactive constituents and pharmaceutical effects. Triterpenoids are important effective ingredients of *G. lucidum*, which are synthesized mainly through the mevalonate pathway. In this study, we aimed to characterize and analyze three committed-step enzymes of the mevalonate pathway, 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase (HMGR), Farnesyl Diphosphate Synthase (FPPS) and Squalene Synthase (SQS), using various bioinformatic tools and servers. As a results, the molecular structures and physicochemical properties of the proteins and their encoding genes (*Glhmgr*, *Glfpps* and *Glsqs*) were studied. The GlHMGR and GlFPPS proteins are localized in the cytosol and lack any transmembrane topological structures. By contrast, GlSQS enters the secretory pathway via its transit peptide and has three transmembrane helices. Phylogenetic analysis suggested that the three proteins could be classified into three large groups according to their significant functional association and genetic conservation. The secondary structures of the GlHMGR, GlFPPS and GlSQS comprise mainly α-helixes and random coils and the three tertiary structures were modeled successfully, including the identification of key motifs. Additionally, the expression levels of the three genes were compared on the basis of dbEST records. In conclusion, the results demonstrated that HMGR, FPPS and SQS from *G. Lucidum* have typical molecular structures and functions. In addition, we inferred that GlFPPS might be the perfect target for genetic engineering because of its position in a bottleneck step, as revealed by metabolic flux analysis.

Keywords: *Ganoderma lucidum*, Triterpenoid, Bioinformatics, Biosynthesis Pathway, Enzyme

Introduction

Isoprenoids are the most abundant natural products, with multiple structures and functions and encompassing more than 30,000 known compounds within the fungus kingdom. As very important metabolites with essential biological functions, isoprenoids are rich in fruit bodies, sporophytes and mycelium to protect these structures from biotic and abiotic stresses, as well as being components of biomembranes and regulators of growth and development (Holstein and Hoh, 2004; Leandro *et al*., 2012; Liang and Guo, 2013). Furthermore, some isoprenoids are economically important chemicals, such as those used as potential drugs, nutraceuticals and industrial materials. As a result, research interest in isoprenoids, especially the economically important ones, has increased significantly.

Isopreoids are divided into several subgroups, including monoterpenoid C_{10}, sesquiterpenoid C_{15}, diterpenoid C_{20} and triterpenoid C_{30}, according to the number of C_5 units in the skeleton. For example, triterpenoids comprise six C_5 units (Liao *et al*., 2006a).

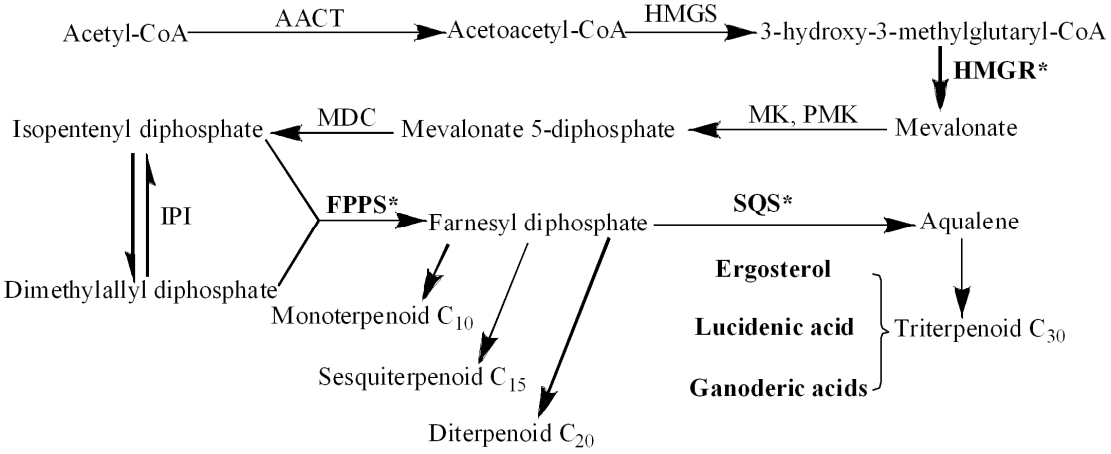

Fig. 1. Triterpenoid biosynthetic pathway in *Ganoderma* cells

All isopreoids are synthesized from two universal precursors: Isopentenyl diphosphate (IPP) and its isomer Dimethylallyl diphospate (DMAPP). However, there are two distinct biosynthesis pathways; the mevalonate (MVA) pathway localized in the cytosol and the deoxyxylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DXP/MEP) pathway in plastids (Dewick, 2002; Aule *et al*., 2003; Chaurasiya *et al*., 2012). The former is predominant in fungal cells and has been studied extensively. The MVA pathway has three successive phases. In the prophase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) catalyzes mevalonate synthesis from HMG-CoA, which is considered the most important committed step of the MVA pathway (Choi *et al*., 1992). Subsequently, in the metaphase, farnesyl diphosphate synthase (FPPS), a representative and crucial enzyme, condenses IPP and DMAPP to form the key direct precursor, Farnesyl diphosphate (FPP) (Enjuto *et al*., 1994). After a branch-point intermediate for various terpenoids based on FPP, Squalene Synthase (SQS) drives the synthesis of triterpenoids, sterols and cholesterols. It is believed that this reaction is also the rate-limiting regulator of triterpenoids biosynthesis (Jennings *et al*., 1991).

Lucidenic acid, ganoderic acid and ergosterol are the predominant economic triterpenoids derived from the well-known Traditional Chinese Medicine *Ganoderma lucidum*, which has potent antitumor activities and is an immunopotentiator employed widely in the treatment of various cancers (Liu *et al*., 2005; Zhang *et al*., 2008; Tello *et al*., 2013). Recently, the synthesis mechanism of the triterpenoid compounds have been studied, as well as the genes encoding the committed enzymes, such as HMGR, FPPS and SQS, which play vital roles in *Ganoderma* triterpenoid biosynthesis (Zhao *et al*., 2007; Ding *et al*., 2008; Shang *et al*., 2008) (Fig. 1). In particular, evidence from genetic engineering and molecular regulation studies showed that these genes are essential targets; however, it is obvious that metabolic pathways act as a network instead of working independently (Fernie, 2003; Liao *et al*., 2006b). Hence, it would be valuable and interesting to investigate these objects simultaneously. In the present study, we performed *in silico* analysis of the HMGE, FPPS and SQS proteins from *G. Lucidum* in terms of their structural properties, biochemical function and expression profiles of their genes, using various bioinformatic tools and servers.

Materials and Methods

Database Analyses

The sequences of the *Glhmgr*, *Glfpps* and *Glsqs* genes, with Complete Coding Regions (CDS) and their corresponding amino acid sequences, were obtained from GenBank and GenPept, respectively: GlHMGR (protein accession no. ABY84848), GlFPPS (protein accession no. ACB37021) and GlSQS (protein accession no. ABF57213).

Information on the Expressed Sequence Tags (ESTs) of *Ganoderma lucidum* (from the GlEST database) was used to identify the key enzymes and their expression levels were obtained using the dbEST records at NCBI. Reads were searched using keywords and BLAST searched against the GlEST database by querying gene products from *G. Lucidum*; those exhibiting an $E_{value} > e^{-10}$ were excluded. The remaining ESTs were clustered according to the bioinformatic indexes.

Comparative Bioinformatic Analyses

Comparative bioinformatic analyses of target sequences were performed online at http://www.ncbi.nlm.nih.gov and http://www.expasy.org. Protparam tools were used to obtain molecular structures and physicochemical properties. Vector NTI

Suite 8 (Lei *et al*., 2009) with default parameters was used to produce multiple alignments of the full-length amino acid sequences. Target P 1.1 Server (Olof *et al*., 2000) predicted the subcellular location and TMHMM Server v.2.0 (Ikeda *et al*., 2002) and ProtScale (Kyte and Doolittle, 1982) analyzed the transmembrane helices and hydrophobicity of the protein, respectively. ScanProsite (Combet *et al*., 2000) and CDD (Marchler and Bryant, 2004) were used to search for motifs and structural domains in the protein. ClustalX (Thompson *et al*., 1997) was used to align the target proteins and their related sequences from other species. Subsequently, the Neighbor-Joining method with 100 replicates was used to construct a phylogenetic tree and the MEGA3 software established the reliability of each node using the bootstrap method (Saito and Nei, 1987; Kumar *et al*., 2001). The CFSSP online tool (Chou and Fasman, 1974; 1975) was used to predict the secondary structures of the three *G. Lucidum* proteins. Swiss-Modeling (Guex and Peitsch, 1997; Schwede *et al*., 2003; Arnold *et al*., 2006) accomplished the homology-based three-dimensional (3D) structural modeling of the target proteins and Web Lab Viewer Lite 4.2 was used to display the 3D structure. Additionally, all the Tentative Consensus (TC) sequences were compared with sequences deposited in public databases. GlEST data were transformed to a relative abundance of 1000 reads/library. The apparent number of specific reads for every target gene with each TC was multiplied by 1000 and then classified by the total number reads for different sets of the gene library. These transformed data were used for expression comparison and graph construction (Adriano *et al*., 2007).

Results

Molecular Structures and Physicochemical Properties

The structural properties of *Glhmgr*, *Glfpps* and *Glsqs* and their encoding proteins, are shown in Table 1. Homology-based 3-D modeling of the target proteins was implemented successfully using SWISS-MODEL on the basis of their own corresponding templates: GlHMGR was based on the catalytic portion of human HMG-CoA reductase with HMG, CoA and $NADP^+$; GlFPPS was based on the structure of human farnesyl pyrophosphate synthetase; and GlSQS was based on the crystal structure of human squalene synthase. As shown in Table 1, the secondary structures of these proteins comprised mostly random coils, α-helices and extended strands, with the first two being the main components. Some important functional motifs were scanned and marked in the tertiary model (Fig. 2).

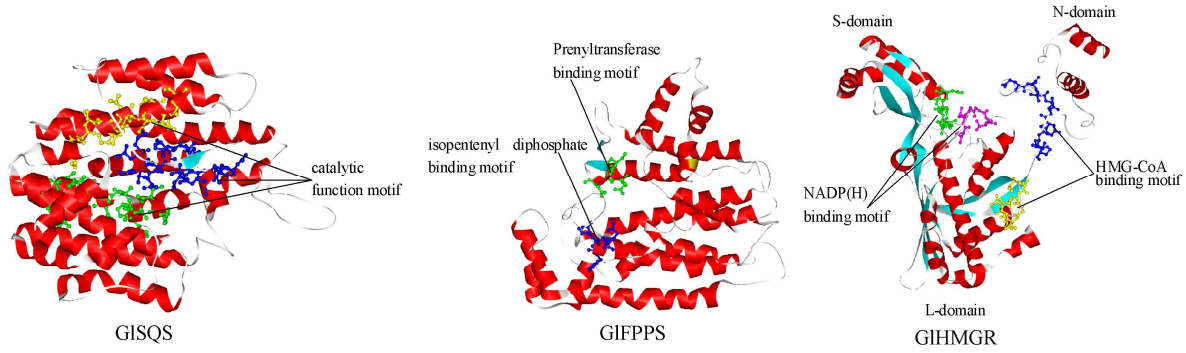

Fig. 2. The 3D structural model was established. The α-helix and extended strand were indicated in red and blue, respectively. Random coil was indicated in silver. Some important motifs were pointed out

Table 1. Analysis of molecular structures and physicochemical properties

Index	HMGR	FPPS	SQS
Amino acid residues (aa)	1226	360	467
Molecular weight (kDa)	131.175	41.014	54.018
PI	8.51	5.34	6.66
Molar extinction coefficient	81330	50560	88975
Formula	$C_{5838}H_{9441}N_{1605}O_{1714}S_{51}$	$C_{1867}H_{2877}N_{485}O_{534}S_{11}$	$C_{2403}H_{3752}N_{676}O_{692}S_{26}$
Instability index	43.13(unstable)	37.65(stable)	47.80(unstable)
Aliphatic index	98.52	92.97	81.28
GRAVY	0.139	-0.194	-0.394
Secondary structure	α-helix: 38.09%	α-helix: 43.89%	α-helix: 42.61%
	β-strand: 16.31%	β-strand: 13.61%	β-strand: 19.06%
	Random coil: 45.60%	Random coil: 42.50%	Random coil: 38.33%

Cytological Characterization and Biochemical Function

The subcellular localization and transmembrane helix prediction suggested that GlFPPS and GlSQS were localized in the cytoplasmic matrix and lacked a signal peptide and transmembrane domain. This indicated that the two enzymes drive substrate conversion and triterpenoid biosynthesis directly, which corresponded with the observation that the MVA pathway synthesizes triterpenoids in the cytoplasmic matrix (Chaurasiya *et al*., 2012). By contrast, GlHMGR was predicted to be located in the secretory pathway because it harbors a 20aa signal peptide and has a three transmembrane region topological structure, which agreed with Campo's results (Campos *et al*., 1995). Sequence analysis revealed that immediately following the signal peptide, GlHMGR has a 70aa plastidial transit peptide. The transit peptide is immediately N-terminal to the catalytic region in the N-terminus, which has three transmembrane helices at A_{13}—A_{35}, A_{222}—A_{244} and A_{251}—A_{270}. The presence of a signal peptide and plastidial transit peptide suggested that HMGR exists exclusively in the Endoplasmic Reticulum (ER).

PROSITE scanning of the amino acid sequences of GlHMGR, GlFPPS and GlSQS identified certain important motifs. Protein kinase C phosphorylation sites and Casein kinase II phosphorylation sites were observed in all three proteins. In addition, GlHMGR and GlFPPS both have N-glycosylation sites, N-myristoylation sites and amidation sites. However, each target protein also has several specific motifs. For example, there are three hydroxymethylglutaryl-coenzyme A reductases signatures in GlHMGR, two polyprenyl synthetases signatures in GlFPPS and one squalene and phytoene synthases signature in GlSQS. It is probable that these signatures play important roles in recognizing and binding the enzymes' substrates. The tool CDD also recognized the presence of conserved domains in these proteins. As shown in Fig. 3, a series of functional domains were detected, such as catalytic residues, substrate binding pockets, metal cofactor binding sites, Asp-rich regions and active site lid residues.

Fig. 3. Secondary structure and conserved domains analysis

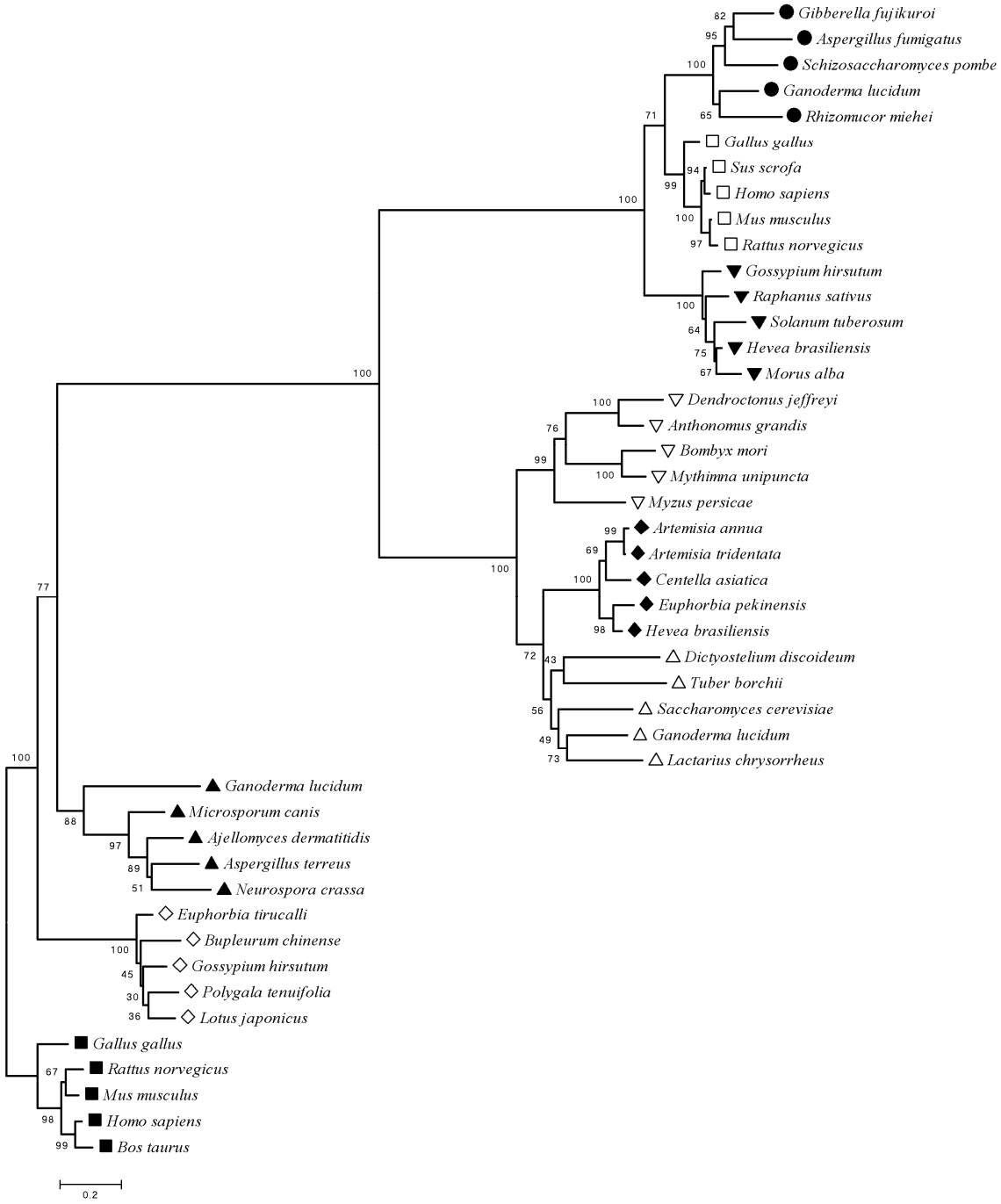

Fig. 4. Molecular phylogram analysis. Phylogenetic tree was constructed by Neighbor-Joining method (based on 1000 bootstrap replicates) using MEGA3 software and the bootstrap values were showed on branches

Multiple Sequence Alignment and Molecular Evolution

After aligning GlHMGR, GlFPPS or GlSQS and the same target protein sequences from other fungal species, we observed that in general, the structural differences in their N-terminal domains probably determined the differences in their activities. Four conserved regions were found in the GlHMGR sequence: Two HMG-CoA binding domains (ENVIGYMP and TAEGTLVA) and two NADP(H) binding domains (DAMGMNM and TVGGGT). Furthermore, a Glu residue was considered as the catalytic center (Wang *et al*., 1990; Lei *et al*., 2008).

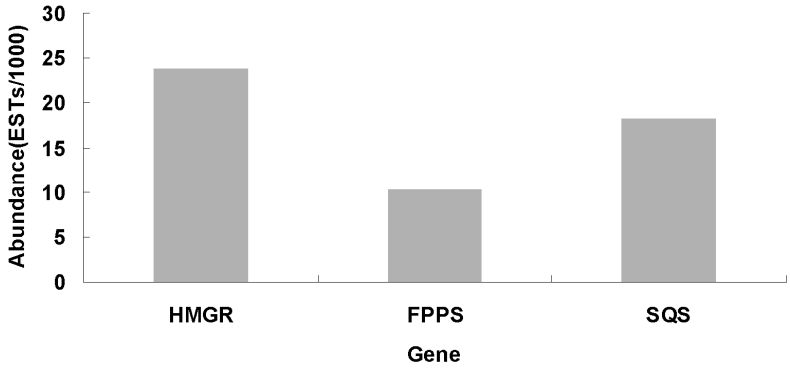

Fig. 5. Expression profiles of *Glhmgr*, *Glsqs* and *Glfpps* genes

Five highly conserved regions were identified in the GlFPPS protein: GKLNR, GQMID, KT, DDMMD and DDFLD. The latter two are Asp-rich motifs (DDXXD), which were reported to be the binding sites of prenyltransferase and its substrate isopentenyl diphosphate (Gerhard, 1994; Ohnuma *et al*., 1996). In GlSQS, domains III, IV, V [YCHYVAGLVGEGLT) RF, GLMLQKTNIIRDFRE, FCAIPQTMAMATLS] are highly conserved, having almost the same sequences in the SQS proteins from almost all fungal species. Thus, the three domains are possibly related to the catalytic function (Robinson *et al*., 1993).

Based on the multiple alignments of 17 protein families by ClustalX, a phylogenetic tree was constructed from various organisms including fungi, animals and plants, using the Neighbor-Joining method (Fig. 4). Intriguingly, HMGRs, FPPSs and SQSs appeared to be derived from a collective ancestor that differentiated gradually into the different groups. The evolutionary positions of HMGR, FPPS and SQS from *G. Lucidum* place them firmly in the fungi groups and are closer to other multicellular than to unicellular fungi.

Comparison of Expression Level

In the GlEST database, approximately 24, 10 and 18 reads identified as *hmgr*-like, *fpps*-like and *sqs*-like records, respectively, were found and clustered into three TCs. Comparison with the dbEST records of NCBI identified ESTs encoding peptides with adequate similarity to the GlHMGR, GlFPPS and GlSQS amino acid sequences (Fig. 5). Based on the read counts for *Glhmgr*, *Glfpps* and *Glsqs*, these TCs showed different levels of expression and inferred the possible involvement of the three committed-step enzymes in triterpenoid biosynthesis and it was obvious that the expression levels, from high to low, were *Glhmgr* > *Glsqs* > *Glfpps*.

Discussion

Based on the differences in their structures and properties, GlHMGR, GlFPPS and GlSQS probably exert their activities in different phases and under different reaction conditions. However, there was some similarity in their physicochemical properties, indicating their functional association in the genetic conservation of triterpenoid biosynthesis pathway.

Ganoderma lucidum is an ancient and valuable mushroom in traditional Chinese medicine and studies on the *hmgr*, *fpps* or *sqs* genes have played a critical role in determining the mechanism of triterpenoid metabolism and its genetic engineering. Investigation and elucidation of their genetic relationships and molecular evolution would be useful to further increase our knowledge of their important functions. According to the comparison of the gene expression levels, these key regulatory genes had somewhat different expressions, designed to meet the diverse requirements of triterpenoid synthesis. Surprisingly, *Glfpps*, encoding the vital regulator of the *Ganoderma* triterpenoid precursor biosynthesis, showed the lowest expression level. Therefore, this key reaction step might be the bottleneck of triterpenoid metabolism and is thus the perfect target of germplasm improvement by genetic engineering.

Conclusion

The medicinal mushroom *Ganoderma lucidum* has been used to prevent and treat various diseases and to promote health and longevity in Asia, especially in China (Yuan *et al*., 2007). Triterpenoids are the main active ingredients and are synthesized by the classical MVA pathway, in which the protein products of the *hmgr*, *fpps* and *sqs* genes are considered as crucial regulators. This study presented the structural properties, biochemical functions and expression profiles of these genes and their corresponding proteins. Three-dimensional modeling and bioinformatics analysis revealed the initial molecular mechanisms and reaction process involving these key enzymes, which will be useful to provide theoretical references for research into the enzymatic properties

and genetic regulation of triterpenoid biosynthesis in *G. Lucidum* and for the development and utilization of this medicinal fungus in the future.

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Author's Contributions

Xiaorong Shui: Performed the study, wrote the manuscript and/or contributed to data analysis and interpretation.

Xiaozhong Lan: Performed the study and/or wrote the manuscript.

Wei Lei: Wrote the manuscript and takes full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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