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Cloning and Characterization of Squalene Synthase (*SQS*) Gene from *Ganoderma lucidum*

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Abstract This report provides the complete nucleotide sequences of the full-length cDNA encoding squalene synthase (SQS) and its genomic DNA sequence from a triterpene-producing fungus, *Ganoderma lucidum*. The cDNA of the squalene synthase (*SQS*) (GenBank Accession Number: DQ494674) was found to contain an open reading frame (ORF) of 1,404 bp encoding a 468-amino-acid polypeptide, whereas the *SQS* genomic DNA sequence (GenBank Accession Number: DQ494675) consisted of 1,984 bp and contained four exons and three introns. Only one gene copy was present in the *G. lucidum* genome. The deduced amino acid sequence of *Ganoderma lucidum* squalene synthase (*Gl-SQS*) exhibited a high homology with other fungal squalene synthase genes and contained six conserved domains. A phylogenetic analysis revealed that *G. lucidum* SQS belonged to the fungi SQS group, and was more closely related to the SQS of *U. maydis* than to those of other fungi. A gene expression analysis showed that the expression level was relatively low in mycelia incubated for 12 days, increased after 14 to 20 days of incubation, and reached a relatively high level in the mushroom primordia. Functional complementation of *Gl-SQS* in a SQS-deficient strain of *Saccharomyces cerevisiae* confirmed that the cloned cDNA encoded a squalene synthase.

Keywords: cDNA cloning, *Ganoderma lucidum*, squalene synthase, functional complement

Ganoderma lucidum (Curtis: Fries) Karsten (Polyporaceae) is widely used in east Asia as a remedy for minor health disorders and to promote vitality and longevity [17]; thus, several studies have recently focused on isolating the pharmaceutically active components to understand the underlying action mechanisms [18, 19]. Over 200 triterpenes have already been isolated from the mushroom [2], some of which display cytotoxic [16], histamine release-inhibiting [10], angiotensin converting enzyme-inhibiting [22], and cholesterol synthesis-inhibiting [11, 28] effects. More recently, several triterpenes have been reported to be active against the human immunodeficiency virus [20, 21]. However, despite the potential medical importance, little is known about the molecular biology of triterpene biosynthesis in *G. lucidum*. As secondary metabolites, triterpenes are synthesized *via* the mevalonate pathway, which involves the sequential conversion of farnesyl diphosphate (FPP) to squalene and then to 2,3-oxidosqualene, followed by a series of cyclization, oxidation, and reduction reactions. This pathway has already been confirmed in *G. lucidum* by isotope labeling experiments [7, 14, 27].

Squalene synthase (farnesyl diphosphate: farnesyl diphosphate farnesyltransferase, EC 2.5.1.21) is a membrane-bound enzyme [23] that condenses two farnesyl diphosphate molecules into squalene. The enzyme is active at the major branch point in the isoprenoid biosynthetic pathway, where FPP can be transformed into ubiquinones, heme A, geranylated proteins, and dolichols [4]. There is also a positive correlation between the expression level of SQS and the amount of triterpenes produced [33]. Thus, as a key enzyme in triterpene biosynthesis, SQS has been cloned from yeast [8, 32], humans [9], rats [30], tobacco [3, 5, 6], *Arabidopsis thaliana* [12, 29], and other species. A comparison of the amino acid sequence of SQS from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, rats, humans, and *A. thaliana*

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has revealed that the overall similarity is relatively low, although some specific regions, presumably involved in substrate binding and enzyme catalysis, are highly conserved among the different enzymes.

Therefore, to gain new insights into the role of SQS in the control of *G. lucidum* triterpene biosynthesis, this study isolated and characterized the full-length cDNA encoding squalene synthase and the putative squalene synthase gene from the fungus.

MATERIALS AND METHODS

Strains and Plasmids

The *G. lucidum*, strain HG, was obtained from the culture collection of the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, and the *S. cerevisiae*, strain 2C1 (*erg*9::*his*3, *ura*3-1, *trp*1-1, *leu*2-1), devoid of any SQS activity, was kindly provided by Dr. F. Karst (Institut National de la Recherche Agronomique, France). *E. coli* strain XL1-blue (Stratagene) was used for the cloning, maintenance, and propagation of the plasmids. The yeast-*E. coli* shuttle vector pYF1845 was kindly provided by Q. H. Yao (Agricultural Academy of Shanghai). The DNA restriction enzyme EcoICRI was purchased from the Promega Corporation, and the other DNA restriction enzymes purchased from the Takara Corporation. The oligonucleotides were synthesized by the Shanghai Sangon Corporation.

The yeast cells were grown at 28°C in either a liquid culture or on agar plates (media supplemented with 15 g agar/l). When required to supplement auxotrophies, ergosterol (4 μg/ml in a liquid culture or 80 μg/ml on agar plates) was added to the growth media, and supplied based on diluting a stock solution (4 mg/ml) in a mixture of Tergitol NP-40/ ethanol (1:1).

Genomic DNA Isolation

The *G. lucidum* mycelia were harvested after 7 days of growth in a potato dextrose broth, frozen in liquid nitrogen, ground using a pestle and mortar, and the genomic DNA isolated using the CTAB method [25].

Construction of cDNA Expression Library

The powdered mushroom primordia were prepared as above, and the total RNA extracted using Isogen (Promega, U.S.A.). The poly(A)$^+$ RNA was then purified using a Straight A's mRNA isolation system (Promega). The RNA concentrations in the samples were measured spectrophotometrically, and a cDNA library constructed using a Zap Express cDNA Gigapack III Gold Cloning Kit according to the manufacturer's (Stratagene) recommendations. The cDNA synthesized from the poly(A)$^+$ RNA was ligated into an EcoRI-XhoI-predigested dephosphorylated Zap Express cloning vector, and then packaged with Gigapack III Gold packaging extracts (Stratagene).

Cloning of Specific Fragment of *G. lucidum SQS* Gene

The amino acid sequences conserved among the *S. cerevisiae*, *S. pombe*, human, and *A. thaliana SQS* genes were used to design two degenerate oligonucleotide primers for the PCR amplification of the *G. lucidum* genomic DNA. The primer sequences were 5'-GA(TC)CA(ATCG)(AT)T(ATCG) GA(AT)GA(TC)GA(TC)ATG-3' (forward primer) encoding the consensus sequence DT(L/V)EDDM in domain II, and 5'-GC(ATCG)AC(AT)TA(AT)TG(AT)CA(AT)TA-3' (reverse primer) encoding the consensus sequence YCHYVA in domain III.

The PCR amplification was carried out using a Programmable Thermal Cycler PTC100 (MJ Research Inc., Watertown, MA, U.S.A.) in 0.5-ml tubes containing 10 μM of each primer, 1 μg of *G. lucidum* genomic DNA, 1×*Taq* DNA polymerase reaction buffer, 0.2 mM of each dNTP, and 2 U of *Taq* DNA polymerase in a final volume of 30 μl (Promega). The amplification conditions were: 30 cycles at 95°C for 30 s, 52°C for 40 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The amplification products were ligated into a pMD18-T vector (Takara), then transformed and sequenced according to standard procedures [26].

Cloning of *G. lucidum SQS* Gene cDNA

Two specific primers (P1: 5'-GAGGATGACATGACGCTT-3' and P2: 5'-ATGGCAGTAGAGGTTGTAC-3') were designed and synthesized based on the sequence of the amplified 395-bp *SQS* gene-specific fragment. The P1 primer was a specific sequence near the 5' end of the gene, and the P2 primer was the complementary sequence near the 3' end of the gene. These two primers were used to screening the cDNA library according to the methods described by Liu *et al.* [15]. Positive clones were used for *in vivo* plasmid excision with an ExAssist help phage and *E. coli* strain XOLOR according to the manufacturer's (Stratagene) protocol. Several clones were subsequently sequenced as above, and the clone containing an apparent full-length ORF was designated pBKS-SQS.

Cloning of Genomic DNA Sequence of *G. lucidum SQS* Gene

Two specific primers were designed and synthesized based on the sequence of the *SQS* cDNA sequence. The primers and sequences were primer LZ1, 5'-ATGGGCGCGA-CGTCTATG-3' (according to the cDNA sequence from 1 to 18 and containing the ATG start codon); and primer LZ2, 5'-TCAGCTCGATGGGGCTTG-3' (complementary to the cDNA sequence from 1,387 to 1,404 and containing the TGA stop codon). Using the *G. lucidum* genomic DNA as the template, the complete *SQS* genomic sequence was obtained by a PCR.

Table 1. Primer sets used for RT-PCR analysis of *Gl-SQS* gene transcription.

Target gene	Primer	Sequence	Predicted product size (bp)
Gl-SQS	Forward	5'-ACAACCCATACTCCGACAATT-3'	222
	Reverse	5'-TTCGTGGTCGCTGCTTTAT-3'	
Gl-GPD	Forward	5'-CTCCTTCACGGAGACATT-3'	340
	Reverse	5'-TAACACCGCAGACGAACA-3'	

Comparison of SQS Protein Sequences

SQS protein sequences were obtained for *S. cerevisiae*, *S. pombe*, *Pichia jadinii*, *Candida albicans*, and *Yarrowia lipolytica* from published reports. The sequences were analyzed for identity using CLUSTAL W and BOXSHADE software and a phylogenetic tree generated by PHILP.

Construction of SQS Recombinant Expression Vector

pYF1845 is a high-copy, autonomously replicating *S. cerevisiae*-*E. coli* shuttle vector that confers ampicillin resistance to *E. coli* and uracil prototrophy to *ura*3 yeast. It also contains a *S. cerevisiae* PGK promoter and TADC1 transcription termination element. The *SQS*-containing pMD18-T vector was digested with SphI, and after blunting the cohesive end, the coding region was obtained by digestion with BamHI. After digestion of the pYF1845 plasmid with BamHI and EcoICRI, the *SQS* gene was cloned into pYF1845 at the BamHI and EcoICRI restriction site, and the resultant expression construct used to transform *E. coli* XL1-blue.

Functional Complementation of *Gl-SQS* in Yeast

S. cerevisiae strain 2C1 (*erg*9::*his*3, *ura*3-1, *trp*1-1, *leu*2-1) (*erg9* is the symbol of squalene synthase) was used to investigate the function of *Gl-SQS*. Strain 2C1 is a mutant yeast strain that lacks *SQS* activity and requires ergosterol for growth. The pYF1845+SQS plasmid was extracted from the *E. coli* host and used for transforming 2C1. The pYF1845+*SQS* transformants were selected on a YPD medium without ergosterol.

Southern Blot Analysis

The genomic DNA was prepared as described above. For a gel blot analysis, 10 μg was digested with various restriction enzymes, separated on a 0.8% (w/v) agarose gel, and transferred to a nylon membrane (Hybond-H$^+$, Amersham) according to the manufacturer's protocol. The membrane was then hybridized with *Gl-SQS* cDNA and washed twice in 2×SSC, 0.1% (w/v) SDS and 0.2×SSC, 0.01% (w/v) SDS at 55°C.

Expression Characteristics of SQS at Different Stages of Development of *G. lucidum*

The primordia and mycelia were collected after incubation for 12, 14, 16, 18, and 20 days. The mycelium from the liquid cultures was handled according to the method of Zhao *et al.* [33].

The relative *Gl-SQS* gene transcription levels were determined using a semiquantitative RT-PCR [1]. The total RNA was isolated from different *G. lucidum* developmental stages using an RNA Isolation Kit (Watson, China) according to the manufacturer's recommendations, and subsequently treated with RNase-free DNaseI (Promega). The total RNA samples (1 μg) were then reverse-transcribed by MMLV reverse transcriptase (Promega) using oligo(dT)$_{17}$ as the

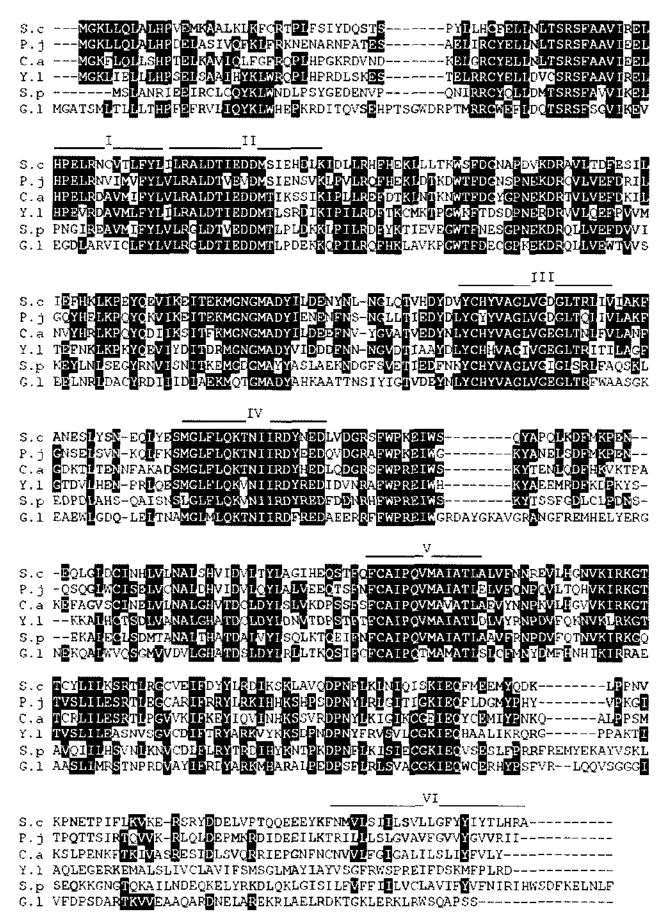

Fig. 1. Comparison of *G. lucidum* SQS amino acid sequence with other fungal SQS sequences.

Data for other fungi were obtained from sequence databases (DDBJ/EMBL/GenBank). Gaps (-) were introduced to maximize the homology, and the residues conserved in at least three sequences are shaded. Six highly conserved peptide domains of 14–23 amino acids reported by Robinson *et al.* [24] are identified. Abbreviations: *S. c*: *Saccharomyces cerevisiae*; *P. j*: *Pichia jadinii*; *C. a*: *Candida albicans*; *Y. l*: *Yarrowia lipolytica*; *S. p*: *Schizosaccharomyces pombe*; *G. l*: *Ganoderma lucidum*.

primer, and the synthesized cDNA used as the template for PCR amplification. The primer sets used for the RT-PCR analysis of the *Gl-SQS* transcripts are shown in Table 1. *G. lucidum* glyceraldehyde-3-phosphate dehydrogenase (*GL-GPD*) gene transcripts were amplified as an internal control [31]. The RT-PCR amplification was carried out as above in 20-μl reaction mixtures.

The amplification conditions were 30 cycles at 94°C for 30 s, 54°C for 40 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The PCR products were size fractionated in 2% (w/v) agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

Cloning of *G. lucidum SQS* cDNA

A 395-bp putative fragment of the *G. lucidum SQS* gene was obtained by a PCR using degenerate primers based on conserved regions, identified using a multiple amino acid sequence alignment of a number of known *SQS* genes. After purification and cloning into pMD18-T, a preliminary sequence analysis and comparison of the deduced amino acid sequence with other reported *SQS* genes confirmed that the cloned sequence encoded part of the *G. lucidum SQS* gene. Use of this sequence to screen a *G. lucidum* expression cDNA library constructed with poly(A)$^+$ RNA isolated from the mushroom primordia generated a clone containing an ORF of 1,404 bases, preceded by 99 bases of the 5' non-translated region (NTR) and followed by 444 bases of the 3' NTR and 17 bases of a poly(A)$^+$ tail. The ORF corresponded to a predicted polypeptide consisting of 468 amino acid residues. The genomic sequence of *Gl-SQS* consisted of 1,984 bp and contained four exons and three introns.

Comparison of Gl-SQS with SQS Sequences from Other Sources

The predicted molecular mass of *Gl*-SQS was 54.01 kDa, which was larger than the 46.9–48.1 kDa range reported for mammalian and higher plant SQSs [3, 24], yet smaller than the SQS from *Ustilago maydis* (64 kDa) (based on GenBank data). The deduced *Gl*-SQS amino acid sequence exhibited a homology with the corresponding enzyme from other fungi, including *U. maydis* (65% similarity, 51% identity) and *S. pombe* (60% and 44%). The results of a multiple alignment analysis of the deduced SQS amino acid sequences from *G. lucidum* and five other fungi are shown in Fig. 1. Six highly conserved peptide domains of 14–23 amino acids (previously pointed out by Robinson *et al.* [24]) were also discernible with the *Gl*-SQS amino acid sequence (Fig. 1). Three domains (III, IV, and V) showed a highly conserved consensus sequence with other SQS enzymes, whereas two domains (I and II) were much less conserved. The carboxyl terminus of the *Gl*-SQS enzyme also contained a putative membrane-targeting domain VI,

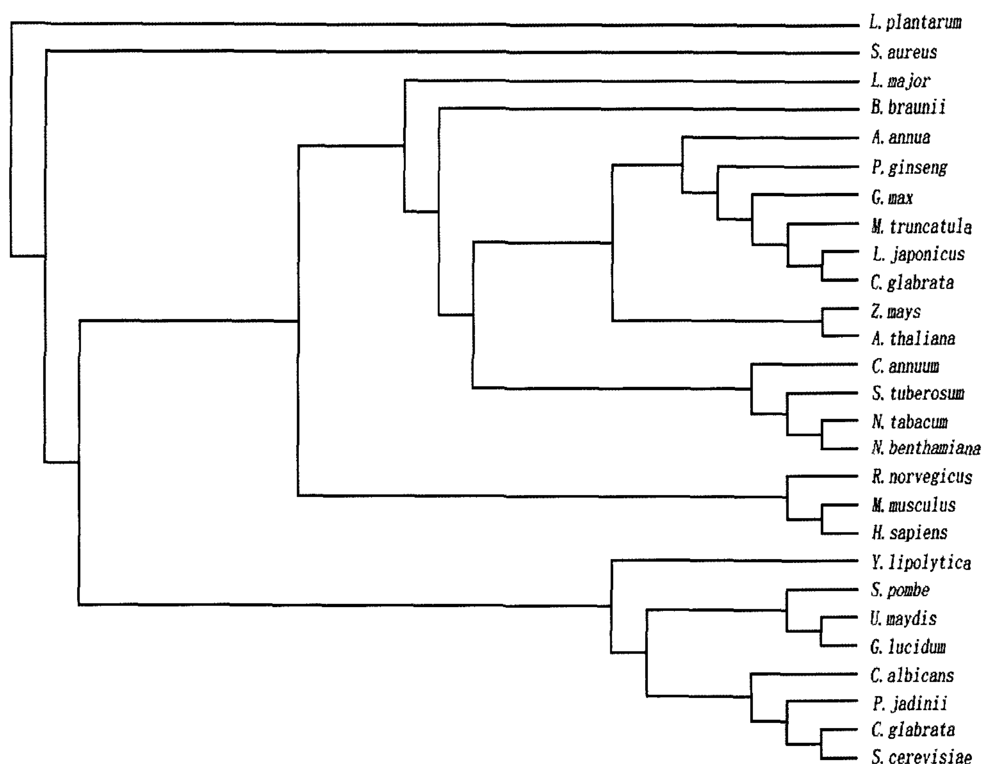

Fig. 2. Phylogenetic analysis of SQS amino acid sequences using the neighbor-joining method.

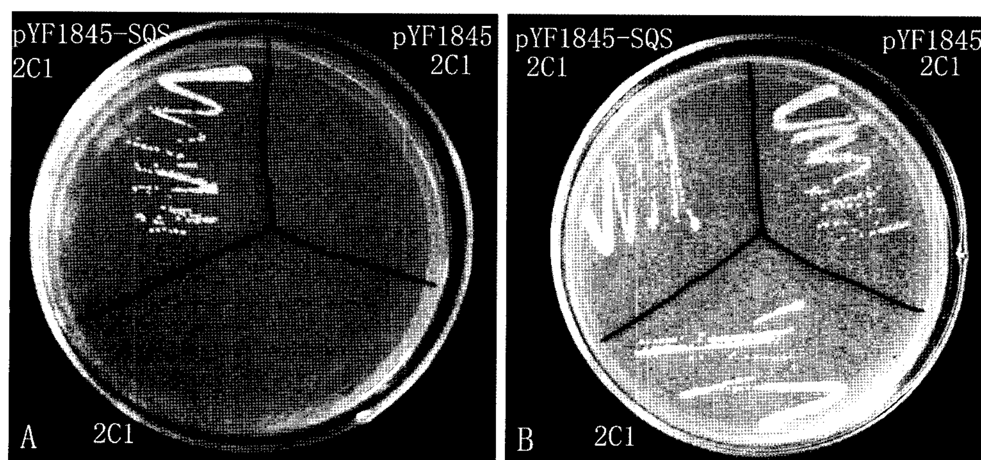

Fig. 3. Functional complementation of *S. cereviseae*, strain 2C1, lacking any SQS activity, with pYF1845+*SQS*.
A. YPD medium without added ergosterol. **B**. YPD medium with ergosterol.

which exhibited a relatively low level of sequence identity with other fungal SQS enzymes. SQS is generally considered to be a crucial branch-point enzyme and potential regulatory point controlling carbon flux into terpenoid synthesis [13, 24]. To our knowledge, this is the first report describing the cloning and analysis of the *SQS* gene from *G. lucidum*.

A phylogentic tree constructed using known SQS amino acid sequences from a wide range of different organisms, including plants, animals, bacteria, protozoa, and fungi, demonstrated that the different forms evolved from a single ancestral gene. *Gl*-SQS was positioned along with other fungal SQS sequences, and more closely related to the SQS of *U. maydis* than to those of other fungi (Fig. 2). This is consistent with the classic diversity of fungi.

Complementation Analysis

Confirmation that *Gl-SQS* was a functional gene encoding squalene synthase was obtained using *S. cereviseae* strain 2C1, a mutant yeast strain lacking SQS activity and auxotrophic for ergosterol. After transformation with the yeast expression vector pYF1845+*SQS*, 2C1 (pYF1845+*SQS*) transformants grew readily on the selective medium (YPD without added ergosterol), whereas the controls 2C1 and 2C1 (pYF1845) (vector only) did not. All three strains grew on the nonselective medium (YPD with ergosterol) (see Fig. 3).

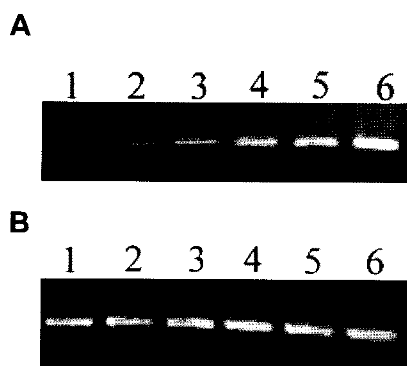

Fig. 4. RT-PCR analysis of *SQS* gene expression at different developmental stages.
A. *SQS*. **B**. *GPD*. Lanes 1–5: 12-, 14-, 16-, 18-, and 20-day-old mycelium, respectively; lane 6: *G. lucidum* primordia.

Southern Blot Analysis

The digestion of *G. lucidum* genomic DNA with BamHI and XbaI, followed by incubation with the full-length *SQS* cDNA revealed a single hybridizing fragment, which is consistent with one gene copy per genome.

Expression of SQS at Different Stages of *G. lucidum* Development

The results of the semiquantitative RT-PCR analysis of the *SQS* gene transcription in the 12–20-day-old fungal mycelia and mushroom primordia are shown in Fig. 4. The gene expression was relatively low in the 12-day-old mycelia, increased after 14 to 20 days, and reached relatively high levels in the primordia. Hirotani *et al.* [7] also observed that the content of triterpenes was much lower in *G. lucidum* mycelia and higher in the fruit body, and a previously reported Western blot data showed that the SQS levels in *G. lucidum* increased during the development of primordia into small fruit bodies [33]. Thus, it was concluded that the lower enzyme expression levels were directly correlated with a low triterpene content, whereas the higher enzyme levels accounted for the increased triterpene synthesis. However, further studies on the character and regulation of *Gl-SQS* are needed to provide more insight into the role of SQS in the control of *G. lucidum* triterpene biosynthesis.

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