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Short communication

Enhanced accumulation of individual ganoderic acids in a submerged culture of *Ganoderma lucidum* by the overexpression of squalene synthase gene

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ABSTRACT

Ganoderic acids (GAs) produced by *Ganoderma lucidum* exhibit antitumor and antimetastasis activities. This study aimed to improve the production of individual GAs by engineering the biosynthetic pathway of GAs in *G. lucidum* through the overexpression of squalene synthase (SQS) gene. SQS catalyzes the first enzymatic step from the mevalonate pathway toward triterpene biosynthesis. The effects of SQS gene overexpression on the accumulation of individual GAs and their intermediates (squalene and lanosterol) by a submerged culture of *G. lucidum* and on the transcription levels of GA biosynthesis genes in this mushroom were investigated. The maximum contents of GA-Mk, GA-T, GA-Me, and GA-S in *G. lucidum* overexpressing the SQS gene were 16, 40, 43, and 53 μg/100 mg dry cell weight, respectively, which were 2.86-, 2.67-, 1.95-, and 1.25-fold of those obtained in wild-type strain (WT). The maximum contents of squalene and lanosterol in the SQS gene-overexpression strain were 1.55- and 1.68-fold higher than those of the WT strain. The transcription levels of the biosynthetic genes encoding SQS and lanosterol synthase were up-regulated by 15.6- and 1.93-fold, respectively, in *G. lucidum* overexpressing the SQS gene, suggesting that increased GA biosynthesis may result from a higher expression of those genes.

1. Introduction

Medicinal mushrooms are rich sources of therapeutically useful and biologically active agents. *Ganoderma lucidum*, a traditional Chinese medicinal mushroom, is used for the prevention and treatment of various human diseases for several thousand years in Asia. Ganoderic acids (GAs) are a type of triterpene compounds produced by *G. lucidum* and have a number of pharmacological activities including cytotoxicity to hepatoma cells, and antitumor, antimetastasis, and anti-HIV activities [1]. Recent studies showed that some individual GAs have interesting bioactivities, for example, GA-S stimulates platelet aggregation [2], GA-T induces apoptosis of lung cancer cells [3], GA-Mk induces apoptosis of HeLa cells [4], and GA-Me inhibits tumor growth and lung metastasis [5,6].

GAs are synthesized via the mevalonate/isoprenoid (MVA) pathway, which involves the sequential conversion of farnesyl diphosphate to squalene, 2,3-oxidosqualene, and lanosterol. The final step after lanosterol formation, including a series of oxidation, reduction, and acylation reactions to form the different individual GAs remain unclear [1]. Some structural genes in the early stages of GA biosynthesis pathway that encode the 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), farnesyl-diphosphate synthase (FPS), squalene synthase (SQS), and lanosterol synthase (LS) have already been isolated and characterized [7].

The submerged culture of *G. lucidum* is considered as a promising alternative for the efficient production of GAs because much time (several months) is usually needed to cultivate the fungal fruiting body, and to control the product quality is difficult [8–10]. Several approaches are adopted to improve the yields of GAs in the submerged culture of *G. lucidum*. These include the manipulation of fermentation conditions, addition of inducers, and development of new bioprocessing strategies [10–17]. However, higher yields of individual GAs in *G. lucidum* mycelia is required for large-scale clinical trials and commercial applications. A highly productive strain is the primary factor to achieve an industrial level GA production. One promising approach to increase triterpene yield is to manipulate the expression levels of the biosynthetic genes [18–20]. An engineered strain for efficient production of GAs may allow for a sustainable and industrial scale production of these valuable metabolites. Our recent work showed that the overexpression of HMGR gene increased total crude GA levels by two folds, but the

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contents of the four major individual GAs (GA-S, GA-T, GA-Me, and GA-Mk) were not affected in *G. lucidum* overexpressing the HMGR gene [21]. From the viewpoint of practical application, GAs exhibit different bioactivities similar to different individuals, therefore, enhancing the accumulation of individual GAs in *G. lucidum* is necessary.

SQS catalyzes the first enzymatic step from the mevalonate pathway toward sterol and triterpene biosynthesis [22]. Overexpression of SQS gene highly increases the accumulation of seven different individual triterpene saponins in *Panax ginseng* and *Eleutherococcus senticosus* [20,23]. In *Withanis somnifera*, the overexpression of SQS gene enhances the levels of two different individual withanolides [24]. These observations indicated that SQS gene is a vital regulatory gene for triterpene biosynthesis. The *G. lucidum* SQS gene was cloned and characterized [25], and its expression had a positive correlation with the triterpene content according to reverse transcription-PCR data [26,27]. However, the regulation of GA biosynthesis by the overexpression of SQS gene remains to be elucidated.

In this study, the effects of the overexpression of SQS gene on the accumulation of GA-S, GA-T, GA-Mk, GA-Me, and their intermediates were investigated using the submerged culture of *G. lucidum*. In addition, the transcription profiles of the three genes encoding the enzymes, namely, HMGR, SQS and LS, were analyzed. This study is helpful for further investigations on the GA biosynthesis regulation and the development of a more efficient fermentation process.

2. Materials and methods

2.1. Strain and culture conditions

G. lucidum CGMCC 5.616 from the China General Microbiological Fermentation Center was maintained on potato dextrose agar slants and cultured at 30 °C for 7 days. The details of preculture medium and conditions were described earlier [26,28]. For the shake-flask fermentation, a 45-ml medium in a 250-ml flask was inoculated with 5-ml of second-stage preculture broth (at an inoculum size of 330 mg dry weight [DW]/L). The culture was incubated in the dark at 30 °C on a rotary shaker at 120 rpm for 12 days. For plasmid construction, *Escherichia coli* strain DH5α was used and grown on Luria-Bertani (LB) agar plates containing 100 μg/ml ampicillin. CYM medium (1% maltose, 2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% $MgSO_4$, 0.46% KH_2PO_4, 0.6 M mannitol, 1% agar) was used for regeneration of protoplasts from *G. lucidum*.

2.2. Vector construction

The plasmid pJW-EXP [29] was used as a backbone to construct the vector pJW-EXP-SQS. The *G. lucidum* SQS gene was amplified from genome DNA using primers SQS-*Nhe*I-F (5′-GCTAGCATGGGCGCGACGTCTATGCT-3′) and SQS-*Sma*I-R (5′-GGGCCCTCACCCGAAAAAGTGGATGAGGAC-3′). The vector pJW-EXP-SQS was made by digesting pJW-EXP with both *Nhe*I and *Sma*I, and inserting the *Nhe*I-*Sma*I PCR fragment containing the *G. lucidum* SQS gene.

2.3. Transformation of G. lucidum and molecular analysis of the transformants

Protoplast formation and transformation of *G. lucidum* were carried out according to the method described by us earlier [29]. The transformants were selected and transferred to fresh CYM medium containing carboxin (2 μg/ml), which was repeated five times to obtain stable transformants. The stable transformants were identified for integration of the fusion fragment of *gpd* promoter and SQS gene by PCR using primers *gpd*-F (5′-GACTTTCTATGTCCGACCTCA-3′) and SQS-R (5′-AGTTGCCTGTCCTTTTCTTT-3′) that should give a 1.87-kb fragment.

2.4. Sampling, analysis of cell dry weight, residual sugar in medium, crude total GAs and individual GAs

Mycelia were harvested by centrifuging a sample at 10,000 × *g* for 10 min, and the precipitated cells were washed for three times with distilled water and then dried at 50 °C to constant weight. The dry cell weight was measured by the gravimetric method. The residual sugar was measured according to the method described by Miller [30]. Crude GAs were extracted and measured according to the method described elsewhere [28]. For determining individual GAs, high-performance liquid chromatography (HPLC) was performed on an Agilent 1200 series (5 μm Agilent Zorbas SB-C18 column, 250 × 4.6 mm), Details on the HPLC analysis method are described elsewhere [26].

2.5. Extraction and analysis of squalene and lanosterol

Cellular squalene and lanosterol were extracted and analyzed according to the method described by Xu et al. [21]. Agilent 1200 series HPLC equipped with an Agilent G1315B diode array detector and an Agilent Zorbax SB-C18 column (250 × 4.6 mm, 5 μm) were used.

2.6. Nucleic acids extraction and cDNA synthesis

The genomic DNA was extracted using the CTAB method, and the total RNA was extracted using TriZol Reagent (Invitrogen, Carlsbad, CA). The quality and quantity of DNA and RNA samples were determined by ethidium bromide stained agarose gel electrophoresis and spectrophotometric measurements. After DNase treatment, 1 μg of total RNA from each sample was reverse-transcribed with RevertAid™ M-MuLV Reverse Transcriptase system for real-time quantitative PCR (qRT-PCR) following the vendor's instructions.

2.7. Measure of hmgr, sqs and ls expression by qRT-PCR

The transcription levels of the *hmgr*, *sqs*, and *ls* (which encode the enzymes HMGR, SQS, and LS, respectively) were analyzed by qRT-PCR. The sequences of the primer for the amplification of *hmgr*, *sqs*, *ls*, and the 18S rRNA gene were described previously [26,31]. The 18S rRNA gene was used as the internal control gene because its expression was found to be stable under our experimental conditions. The expression level of the different genes was normalized with respect to the 18S rRNA gene expression level. For each gene, an expression level of 1 was assigned to the samples from the wild-type (WT) strain, and the expression levels in the *G. lucidum* overexpressing the SQS gene are presented as the fold changes relative to this reference level. Post qRT-PCR calculations to analyze relative gene expression were performed according to the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen [32].

2.8. Statistical analysis

Data are the averages of three independent sample measurements. The error bars indicate the standard deviations from the means of triplicates. The data were analyzed with Student's *t* test. The difference between contrasting treatments was considered significant when p was <0.05 in a two-tailed analysis.