Enhancing ergosterol production in *Pichia pastoris* GS115 by overexpressing squalene synthase gene from *Glycyrrhiza uralensis*

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**Abstract** The present study was designed to determine the effects of copy number variations (CNVs) of squalene synthase 1 (SQS1) gene on the mevalonate (MVA) pathway. SQS1 gene from *G. uralensis* (GuSQS1) was cloned and over-expressed in *Pichia pastoris* GS115. Six recombinant *P. pastoris* strains containing different copy number of GuSQS1 were constructed. HPLC was used to assay the level of ergosterol in all transgenic *P. pastoris* strains containing GuSQS1. HPLC analysis showed that the contents of ergosterol in all of the transgenic *P. pastoris* containing GuSQS1 were higher than that in the negative control. And with the increase of copy number of GuSQS1, the content of ergosterol showed an increasing-decreasing-increasing pattern. The contents of ergosterol in 10-copy-GuSQS1 *P. pastoris* and 47-copy-GuSQS1 *P. pastoris* were significantly higher than that in the rest recombinant *P. pastoris* strains. In conclusion, the CNVs of GuSQS1 influence the content of secondary metabolites in the MVA pathway. The present study provides a basis for over-expressing GuSQS1 and increasing the content of glycyrrhizin in *G. uralensis* cultivars.

**Keywords** Glycyrrhiza uralensis; GuSQS1; Over-expression; Pichia pastoris; Copy number variations

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**Introduction**

*Glycyrrhiza uralensis* Fisch (commonly named licorice) is a well-known medicinal plant in China. The root of *G. uralensis* is widely used in traditional Chinese medicine (TCM) for their functions of relieving coughing, nourishing *qi*, tonifying spleen and stomach, alleviating pain, and eliminating phlegm [1-2]. In addition, it is also used in many other fields such as health food, flavoring agent, commodity, and tobacco additives. *G. uralensis* contains various natural active components, which are responsible for numerous applications and potent effects. Among the active components, glycyrrhizin is the marker compound to characterize the quality of this Chinese herb [3]. In recent years, many studies have demonstrated various biological activities of glycyrrhizin such as anti-tumor, anti-inflammatory, and immune-stimulating activities [5-7].

Wild *G. uralensis* resources have shown a progressive decline, which is caused by irresponsible excessive exploitation. The Chinese government has recently imposed a serial of restrictions on the collection and commercial uses of wild *G. uralensis* (National Development and Reform Commission [2000] No. 13). As a result, *G. uralensis* cultivars have become the main resource of this Chinese herb. However, the level of glycyrrhizin in *G. uralensis* cultivars is remarkable low and cannot meet the requirements of Chinese pharmacopoeia [8]. So improving the quality of cultivars becomes a key issue. Some researchers have tried to solve this problem by accumulating glycyrrhizin in cell suspension cultures of *G. uralensis* [9-12]. However, the results are not promising; glycyrrhizin is not present in cell suspension cultures of *G. uralensis*. Recent report about genetic engineering in *G. uralensis* is infrequent, but using genetic engineering method to increase the content of secondary metabolites in other plants is very common [13-14]. Therefore, developing genetic engineering method to increase the content of glycyrrhizin in *G. uralensis* would be important and interesting. And one of the effective ways is to modify the biosynthetic pathway of glycyrrhizin.

In the biosynthetic pathway of glycyrrhizin (MVA pathway), squalene synthase (SQS) is considered to play an important role [15-16], as an up-regulator for the production of triterpenoids [16-19]. SQS gene belongs to a multigene family; two kinds of SQS with different expressing activity have been
Materials and Methods

Construction of the yeast expression vectors

There are 4 restriction enzyme cutting sites, SnaB I, EcoR I, Aver II and Not I in multiple clone site (MCS) of pPIC9K (Fig. 1). After analyzing GuSQS1 gene sequence using Primer Premier 5.0, we selected Not I and SnaB I as the specific enzyme sites to insert SQS1 gene. Primer pairs with the specific enzyme sites, which are shown in brackets and underlined, are as follows:

**SF:** 5′-CGGTACGTAATGGGGAGTTTGGGAGCGAT-3′ (SnaB I)

**SR:** 5′-ATACGCGGCCGCGTGTGGATGATGCTG-3′ (Not I)

Recombinant plasmid containing GuSQS1 was kept in E. coli DH5α cells in molecular pharmacognosy laboratory, Beijing University of Chinese Medicine, Beijing, China. It was extracted by plasmid extraction kit (Beijing Biomed Medical Technology Co., Ltd., Beijing, China) and used as PCR template. The PCR cycling parameters were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, annealing at 64 °C for 30 sec, extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. The amplified fragments were purified and subcloned into pMD19-T (Takara, Otsu, Shiga, Japan). The resultant vector, GuSQS1-T, was digested with SnaB I (2 h at 37 °C) and Not I (2 h at 37 °C), and then subcloned into pPIC9K (Invitrogen, Carlsbad, California, USA). The resultant recombinant plasmid, pPIC9K-GuSQS1, was transferred into the disarmed E. coli DH5α [32], and then sequenced for correct insertion.
pMD19-T and then transformed into disarmed E.coli DH5α. Thestandard plasmid pMD19-T-GuSQS1 and pMD19-T-GAP were obtained and diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 copy number/2 µL. The primer pairs listed in Table 1 were used in real-time PCR analysis. The real-time PCR cycling parameters were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and storing at 4 °C. By Ct value as X-axis and log value of concentration of standard plasmid as Y-axis the standard curve was established. All of the transgenic P. pastoris were amplified respectively in the same conditions of real-time PCR. The ratio between Y value of GuSQS1 gene and GAP gene was used as the copy number of GuSQS1 gene in each strain of the recombinant P. pastoris.

| Table 1 Primers used in real-time PCR |
|---------------------------------------|
| Gene       | Primers                        |
| GAP        | rGF: 5′-GGTATTAACGCTTCCGACGTATTG-3′ |
|            | rGR: 5′-GATGGTGACAGGGTCTCTCTTTG-3′ |
| GuSQS1     | rSF: 5′-GTGTTCCACCTGCTATCAG-3′     |
|            | rSR: 5′-CGGCACCTTGACATCAGTAG-3′     |

Semi-quantitative RT-PCR analysis
Using yeast RNA rapid extraction kit (Beijing Biomed Medical Technology Co., Ltd, Beijing, China) total RNA was isolated from different strains of the recombinant P. pastoris, RNase-free DNase I enzyme (Tiangen Biotech Co., Ltd, Beijing, China) was used to remove plasmid DNA according to the manufacturer’s instructions. The concentration of RNA was determined by spectrophotometry. The cycle number was set as 18, 20, 22, 24, 26, and 28, respectively, and the optimal cycle number was determined by electrophoresis in 1% (W/V) agarose gel. The RT-PCR cycling parameters were as follows: 50 °C for 30 min; 94 °C for 2 min; optimal cycles of 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 45 sec; a final extension at 72 °C for 10 min; and storing at 4 °C. The paired primers, GF and GR, mentioned above were used to amplify GAP gene as an internal control.

Determination of ergosterol in different strains of recombinant P. pastoris
HPLC analysis was performed on a Waters 2695 system (Waters Corporation, Miford, Massachusetts, USA), equipped with DAD. A Phenomenex LUNA C18 column (250 mm × 4.6 mm, 5 µm, (Phenomenex, Torrance, California, USA)) was applied, and an isocratic elution system consisted of methanol and water (97 : 3) was used. The flow rate was 1.0 mL·min⁻¹ and the column temperature was set at room temperature. The injection volume was 20 µL. The UV detection wavelength was set at 283 nm.

Standard substance ergosterol (11.38 mg, purity: 97.7%) was placed into a 10-mL volumetric flask and diluted with ethanol to volume as reference stock solution. A series of standard solutions were prepared at six concentrations of ergosterol (representing 0.01%, 0.05%, 0.1%, 0.5%, 2%, and 5%) by dilution of the above reference stock solution using ethanol. The calibration curve of ergosterol was obtained by plotting the peak areas versus the concentrations. The precision was detected with the 0.1% standard solution (n = 6), and relative standard deviation (RSD) was calculated.

Limit of detection (LOD) and limit of quantity (LOQ) were determined with a series of dilute solutions of reference stock solution. In order to evaluate the accuracy of the HPLC method, the recovery was analyzed. Nine samples of P. pastoris GS115 without the recombinant plasmid were accurately weighed (50 mg) and divided into 3 groups. 2.91, 5.05, and 8.41 µg of reference substance ergosterol were added into each sample of the three different groups, respectively. Average recovery and RSD values were calculated.

Recombinant P. pastoris containing different copies of GuSQS1 gene were induced to express, and P. pastoris GS115 without the plasmid pPIC9K-GuSQS1 was used as the negative control. All the samples of 96-h culture were collected by centrifugation (5 000 r·min⁻¹, 5 min) and lyophilized. Dried powder of each sample (50 mg) was accurately weighed and extracted with 8 mL of ethyl acetate by ultrasonic extraction for 1 h. The ethyl acetate was evaporated to dryness, and the residue was dissolved in 2 mL ethanol, 20 µL of which was injected onto HPLC for analysis.

Results
Construction of the yeast expression vectors
A 1372 bp fragment was obtained by PCR, and BLAST results showed that it was 98% homologous to the sequence of GuSQS1 gene (GQ266154.1), which demonstrated that the fragment was GuSQS1 gene. It was inserted to the SnaB I-Nor I site of pPIC9K, and the recombinant plasmid pPIC-GuSQS1 was obtained. Figs. 2 and 3 show the constructions of pPIC-GuSQS1. In Fig. 2, lane 1 is the marker, lanes 2–3 are fragments obtained by PCR, which have the correct length. In Fig. 3, SQS represents the sequence of GuSQS1 gene registered in GenBank (HM0 12832.1), and SP1 represents the sequence of fragment cloned from pPIC-GuSQS1, indicating that they were identical.

Construction of recombinant P. pastoris
The linearized pPIC-GuSQS1 was transformed into the disarmed P. pastoris GS115. Single colonies grown on MM and MD plates simultaneously can use methanol in their life cycle, demonstrating the correct insertion. PCR results showed that most of the colonies were positive (Fig. 4). After inducing for 96 h, the difference between the control and recombinant P. pastoris was obvious: the color of negative control was dark yellow, while the color of recombinant P. pastoris with GuSQS1 gene was yellowish-white. The SDS-PAGE result showed there was a 47 kDa-band in recombinant P. pastoris, which was not present in the negative control. All these results proved that the construction of recombinant P. pastoris containing GuSQS1 gene and the expression of GuSQS1 gene was successful.
Copy numbers of GuSQS1 gene in transgenic P. pastoris

PCR and sequencing results showed that the standard plasmids were correct. The melting curves of GAP and GuSQS1 were both unimodal, which suggested that primers used in real time PCR were specific. The standard curve of GAP was as follows: $Y = -2.994X + 35.27$ ($R^2 = 0.997$), and the standard curve of GuSQS1 was as follows: $Y = -2.747X + 33.78$ ($R^2 = 0.997$). Copy numbers of GuSQS1 in different transgenic P. pastoris strains are listed in Table 2. In the present study, six different recombinant P. pastoris strains containing 1, 2, 4, 10, 16 and 47 copies of GuSQS1 gene, respectively, were successfully constructed.

Semi-quantitative RT-PCR analysis of recombinant P. pastoris

The RT-PCR cycle number of GAP was finally fixed to be 20, and the RT-PCR cycle number of GuSQS1 was finally fixed to be 24, according to the results of preliminary experiments. RT-PCR analysis showed that GuSQS1 expressed in all transgenic P. pastoris at different levels (Fig. 5).

As shown in Fig. 6, the relative expression of GuSQS1 in recombinant P. pastoris containing 4-copy-GuSQS was higher than that in the rest of recombinant P. pastoris strains.

ergosterol production in different recombinant P. pastoris strains

The HPLC method was validated according to linearity, sensitivity, precision, stability and accuracy. The retention time of ergosterol in HPLC was 8.157 min. A representative
Table 2  Copy numbers of *GuSQS1* in different recombinant *P. pastoris*

| No. of strains | Average Ct value | Y value in standard curve | Copy numbers of *GuSQS1* 

(GuSQS1/GAP) |
|---------------|------------------|---------------------------|---------------------------|
|               | *GAP*            | *GuSQS1*                  |                           |
| 1             | 23.12            | 22.56                     | 1.1 × 10^4                 | 1.2 × 10^4                 | 1 |
| 2             | 17.44            | 16.43                     | 0.9 × 10^6                 | 2.1 × 10^6                 | 2 |
| 3             | 18.49            | 16.57                     | 4.0 × 10^5                 | 1.8 × 10^6                 | 4 |
| 4             | 17.95            | 15.03                     | 6.1 × 10^5                 | 6.7 × 10^6                 | 10 |
| 5             | 20.16            | 16.60                     | 1.1 × 10^7                 | 1.8 × 10^6                 | 16 |
| 6             | 26.35            | 20.98                     | 9.5 × 10^2                 | 4.6 × 10^4                 | 47 |

Fig. 5  Semi-quantitative RT-PCR results in different recombinant *P. pastoris*

A represents RT-PCR results of *GAP*; B represents RT-PCR results of *GuSQS1*; and the numbers on the picture represent the copy number of *GuSQS1* in the recombinant *P. pastoris*

![Fig. 5](image)

Fig. 6  The expression of *GuSQS1* in different recombinant *P. pastoris* strains

The standard curve for ergosterol was as follows: \( Y = 3.818 \times 10^{-5}X - 7.930 \times 10^{-3} \) \( (R^2 = 0.9999) \). And it had good linearity between 0.1 \( \mu g \cdot mL^{-1} \) to 50.0 \( \mu g \cdot mL^{-1} \). The LOQ (S/N of 10) was less than 2.224 ng and the LOD (S/N of 3) was less than 0.889 ng; RSD value of precision was 0.059 2% \( (n = 6) \), which demonstrated that the established method was precise. The average recovery was 97.28%–101.04%, and RSD value of recovery was 0.548%–0.822%. All the above results demonstrated that this method were acceptable for analysis of the contents of ergosterol in samples.

The contents of ergosterol in all the samples are shown in Fig. 7. The statistical analysis results with independent-sample *T* test results \( (n = 3) \) are listed in Table 3. The contents of ergosterol in all recombinant *P. pastoris* containing *GuSQS1* were 1.15–1.96 times higher than that in the negative control. And with the increase in the copy number of *GuSQS1*, the contents of ergosterol firstly increased, then decreased, and finally increased again. When the copy number was 1 or 2, the contents of ergosterol were similar. But when

![Fig. 7](image)

Table 3  Independent-sample *T* test results of the content of ergosterol in different recombinant *P. pastoris* \( (n = 3) \)

|   | 0   | 1   | 2   | 4   | 10  | 16  | 47  |
|---|-----|-----|-----|-----|-----|-----|-----|
| 0 | 0.036 | 0.046 | 0.005 | 0.000 | 0.001 | 0.000 |
| 1 | 0.036 | 0.780 | 0.035 | 0.000 | 0.002 | 0.000 |
| 2 | 0.046 | 0.780 | 0.027 | 0.000 | 0.002 | 0.000 |
| 4 | 0.005 | 0.035 | 0.027 | 0.000 | 0.030 | 0.000 |
| 10 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.347 |
| 16 | 0.001 | 0.002 | 0.002 | 0.030 | 0.000 | 0.000 |
| 47 | 0.000 | 0.000 | 0.000 | 0.000 | 0.347 | 0.000 |

Note: In this table, the following numbers 0, 1, 2, 4, 10, 16, 47 represented the blank *P. pastoris* and the different recombinant *P. pastoris*
strains containing 1, 2, 4, 10, 16 and 47 copies of GuSQS1 genes, respectively. The copy number was 10, the content of ergosterol reached the highest (about 2.0 times of negative control). Then when the copy number increased to 16, the content of ergosterol did not increase but decrease (only 1.4 times of negative control). However, when the copy number substantially increased to 47, the content of ergosterol increased again and reached to 1.9 times (25.21 μg) of the negative control. Therefore, the copy number of GuHMGR influenced the level of ergosterol in transgenic P. pastoris.

Discussion

The roots of G. uralensis are widely used in many fields in China. There is an irreconcilable conflict between large market demand and low content of glycyrrhizin in G. uralensis cultivars. How to increase the accumulation of glycyrrhizin in G. uralensis cultivars is an important issue. In our previous researches, we found that CNVs of functional genes, such as SQS and HMGR, were present in G. uralensis cultivars. Therefore, it is meaningful and interesting to analyze the relationship between CNVs of functional genes and the content of glycyrrhizin in G. uralensis.

In the present study, we constructed six strains of recombinant P. pastoris containing different copy numbers of GuSQS1. Using real-time PCR, the copy numbers of GuSQS1 in different transgenic P. pastoris were 1, 2, 4, 10, 16, and 47 copies, respectively. The RT-PCR results showed that GuSQS1 was expressed in transgenic P. pastoris at different levels. And the relative expression of GuSQS1 in recombinant P. pastoris containing 4-copy-GuSQS1 was higher than that in other recombinant P. pastoris. Because SQS1 gene is also an important gene in the self-metabolism of P. pastoris, so the analysis of metabolites of P. pastoris can help better understand the influence of CNVs of GuSQS1 in the biosynthetic pathway. The HPLC results showed that, in all the recombinant P. pastoris, the contents of ergosterol were greater than that in the negative control. And with the increasing of copy number of GuSQS1 the content of ergosterol presented in an increasing-decreasing-increasing pattern. In the recombinant P. pastoris containing 10 copies and 47 copies of GuSQS1 gene the contents of ergosterol were much higher than that in others.

Biologically, the difference in gene copy number can lead to variations of enzyme content and finally affect the formation of relevant products. Accordingly, with the increase of GuSQS1 copy number, the content of corresponding enzyme was also increasing, resulting in the accumulation of ergosterol. As a result, the content of ergosterol in recombinant P. pastoris containing 10 copies of GuSQS1 was higher than that in recombinant P. pastoris containing 1 copy, 2 copies and 4 copies of GuSQS1. However, when the copy number increased to 16, the content of ergosterol did not increase, but decrease. Regarding this phenomenon, we can only conjecture how this was happening. Since the expression of GuSQS1 in recombinant P. pastoris containing 16-copy-GuSQS1 gene was not the lowest, the chance of gene silencing caused by integration sites of exogenous gene was very small. Recently many researchers have reported the feedback inhibition in plants over-expressing exogenous genes. So it is possible that increasing of squalene synthase and 2, 3-oxidosqualene result in feedback inhibition of the upstream steps in MVA pathway and finally influence the accumulation of ergosterol. However, when copy number increased to 47, the content of ergosterol (25.21 μg) increased again and was near to the highest level (26.05 μg) under the experimental conditions. Because there are still uncertainties about the complicated procedure of MVA pathway, this phenomenon remained to be elusive. We speculate that the possible reason is the interruption of the feedback inhibition. In organisms when the decrease in enzyme level caused by feedback inhibition to some extent, the feedback inhibition will be inhibited and the metabolic pathway will be activated. This may be the reason of the high content of ergosterol in P. pastoris containing 47 copies of GuSQS1 gene. At the same time, there is another explanation for this, in recombinant P. pastoris such an abundant expression of an exogenous gene may cause a great burden in the self-metabolism of host strain, which may cause the disorder of biosynthetic pathway of ergosterol in P. pastoris containing too many GuSQS1 genes. Nevertheless, the mechanisms responsible for the relations between copy number and gene products need further investigations.

In summary, the present study determined that there was a close relationship between CNVs of GuSQS1 gene and the content of ergosterol. As a key enzyme in MVA pathway, it can also increase the accumulation of glycyrrhizin in G. uralensis. However, the formation of glycyrrhizin in G. uralensis is a very complicated metabolic network and regulated by many enzymes, and GuSQS1 is only one of them. This study provided an important basis for further work on exploring the biosynthesis of glycyrrhizin in vitro. In addition to G. uralensis some other Chinese herbs, such as Glycyrrhiza glabra also harbor glycyrrhizin. Therefore, this study could form basis for further studies on glycyrrhizin biosynthesis in those medicinal plants.

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| BMGY | buffered glycerol-complex medium |
| BMMY | buffered methanol-complex medium |
| CNVs | copy number variations |
| HPLC | high-performance liquid chromatography |
| LOD | limit of detection |
| LOQ | limit of quantity |
| MCS | multiple clone site |
| MD | minimal dextrose medium |
| MM | minimal medium |
| MVA | mevalonic acid |
| RSD | relative standard deviation |
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