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

LIU Ying[1], ZHU Xiao-Qing[1], LI Wen-Dong[2], WEN Hao[1], LIU Chun-Sheng[1]*

[1]*School of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing 100102, China;*
[2]*Beijing Institute for Drug Control, Beijing 100035, China*

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

[KEY WORDS] *Glycyrrhiza uralensis*; *Gu*SQS1; 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 [2]. In recent years, many studies have demonstrated various biological activities of glycyrrhizin such as anti- tumor, anti-inflammatory, and immune-stimulating activities [3-7].

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 can not 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

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[*Corresponding author] Tel: 86-10-84738624, Fax: 86-10-84738611, E-mail: max_liucs@263.net
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found in *Arabidopsis thaliana* [20-21], *Glycyrrhiza glabra,* [22] and several other plants [23-24]. In the present study, *SQS*1 was selected as a target gene. We hypothesized that overexpressing *SQS*1 gene from *G. uralensis* (*Gu*SQS1) may affect the accumulation of glycyrrhizin. There are few reports on the transformation and overexpression of *Gu*SQS1 gene. Lu *et al* [17] and Zhang *et al* [25] have investigated the overexpression of *Gu* SQS1 gene in hairy root cultures of *G. uralensis*. However, the effects of copy number of *Gu*SQS1 gene in transgenic hairy roots on the production of metabolites were not studied in their work.

Copy number variations (CNVs) include deletions, duplications, insertions, and more complex variations ranging from 1 kb to submicroscopic sizes [26]. As a new type of genomic variation, CNVs have received considerable attention in recent years. Many characteristics of CNVs, such as wide distribution, hereditability, relative stability, and high heterogeneity have been reported [26-27]. CNVs can lead to the change in gene dosage and further phenotype character. There is a close relationship between CNVs and human diseases [28-31]. However, there are few reports about CNVs in plants. In the present study, we determined the relationship between CNVs of *Gu*SQS1 gene and the level of ergosterol. It was hoped that our study would provide a basis for overexpression of *Gu*SQS1 gene and increasing the accumulation of glycyrrhizin in *G. uralensis* cultivars.

Materials and Methods

Construction of the yeast expression vectors

There are 4 restriction enzyme cutting sites, *SnaB* I, *EcoR* I, *Avr* II and *Not* I in multiple clone site (MCS) of pPIC9K (Fig. 1). After analyzing *Gu*SQS1 gene sequence using Primer Premier 5.0, we selected *Not* I and *SnaB* I as the specific enzyme sites to insert *SQS*1 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′-ATAGCGGCCGCCGTGTTTGACCATTCGTTTC-3′ (*Not* I)

Recombinant plasmid containing *Gu*SQS1 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, *Gu*SQS1-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, Carisbad, California, USA). The resultant recombinant plasmid, pPIC9K-*Gu*SQS1, was transferred into the disarmed *E.coli* DH5*α* [32], and then sequenced for correct insertion.

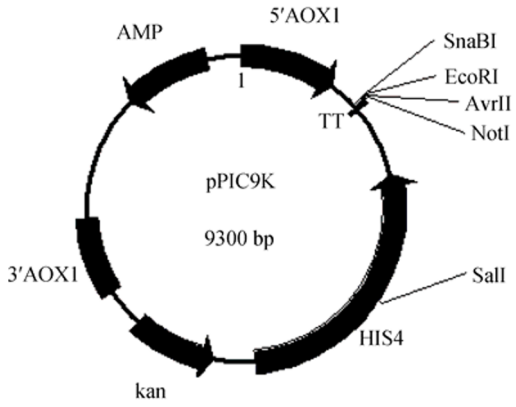

Fig. 1 Structure of pPIC9K

Construction of recombinant P. pastoris

pPIC9K-*Gu*SQS1 was linearized by restriction enzyme *Sal* I and mobilized into the disarmed *P. pastoris* GS115 (Invitrogen) by electroporation (1 500 V, 25 μF, 400 Ω). Then 0.5 mL of yeast peptone dextrose medium (YPD) was added into the production of electroporation, which was cultured at 30 °C, 200 r·min^{-1} for 1 h. 200 μL of the above suspension culture were incubated on minimal dextrose medium (MD) plates at 30 °C for 48 h. Single colonies were selected and incubated on minimal medium (MM) and MD plates at the same time at 30 °C for 2−4 d; and the colonies grown on both MM and MD mediawere selected.

PCR was used to check the correction of recombinant *P. pastoris* containing *Gu*SQS1 gene [33]. The primers used were as follows: forward primer: 5′-TACTATTGCCAGCATTGCTGC-3′; reverse primer: 5′-GCAAATGGCATTCTGACATCC-3′. The PCR cycling parameters were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min.

The selected recombinant *P. pastoris* were induced to express *Gu*SQS1 gene (medium: buffered glycerol-complex medium (BMGY) and buffered methanol-complex medium (BMMY), 30 °C, 250 r·min^{-1}). The *P. pastoris* containing a void vector was used as the negative control. After a 96-h induction, the supernatant was examined by SDS-PAGE (12% resolving gel, 5% stacking gel) using Coomassie brilliant blue staining.

Copy number determination

GAP gene from *P. pastoris* was selected as internal control gene in real-time PCR [34]. Primers of *GAP* (Genbank accession number: U62648) were as follows: GF: 5′-CACAATGGCTATCACTGTCG-3’; GR: 5′-GACACACTACAGCCCGCATT-3’. Primers of *Gu*SQS1 gene were as follows: SF: 5′-CGGTACGTAATGGGGAGTTTGGGAGCGAT-3′; SR: 5′-ATAGCGGCCGCCGTGTTTGACCATTCGTTTC-3′. The PCR cycling parameters were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. The amplified fragments were subcloned into

pMD19-T and then transformed into disarmed *E.coli* DH5α. Thestandard plasmid pMD19-T-*Gu*SQS1 and pMD19-T-GAP were obtained and diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 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 *Gu*SQS1 gene and *GAP* gene was used as the copy number of *Gu*SQS1 gene in each strain of the recombinant *P. pastoris*.

Table 1 Primers used in real-time PCR

Gene	Primers
GAP	rGF: 5′-GGTATTAACGGTTTCGGACGTATTG-3′
	rGR: 5′-GATGGTGACAGGGTCTCTCTCTTGG-3′
GuSQS1	rSF: 5′-GTTTCGCACTCGTCATTCAG-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 manufacture'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 C_{18} 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 ethnol. 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 *Gu*SQS1 gene were induced to express, and *P. pastoris* GS115 without the plasmid pPIC9K-*Gu*SQS1was 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 *Gu*SQS1 gene (GQ266154.1), which demonstrated that the fragment was *Gu*SQS1 gene. It was inserted to the *SnaB* I-*Not* I site of pPIC9K, and the recombinant plasmid pPIC-*Gu*SQS1 was obtained. Figs. 2 and 3 show the constructions of pPIC-*Gu*SQS1. 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 *Gu*SQS1 gene registered in GenBank (HM0 12832.1), and SP1 represents the sequence of fragment cloned from pPIC-*Gu*SQS1, indicating that they were identical.

Construction of recombinant P. pastoris

The linearized pPIC-*Gu*SQS1 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 *Gu*SQS1 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 *Gu*SQS1 gene and the expression of *Gu*SQS1 gene was successful.

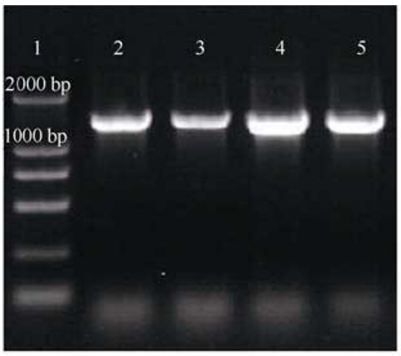

Fig. 2 PCR results of recombinant plasmid pPIC-*Gu*SQS1 Lane 1 is the marker; lanes 2−5 are fragments obtained by PCR

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 *Gu*SQS1 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 *Gu*SQS1 was as follows: $Y = -2.747X + 33.78$ (R^2 = 0.997). Copy numbers of *Gu*SQS1 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 *Gu*SQS1 gene, respectively, were successfully constructed.

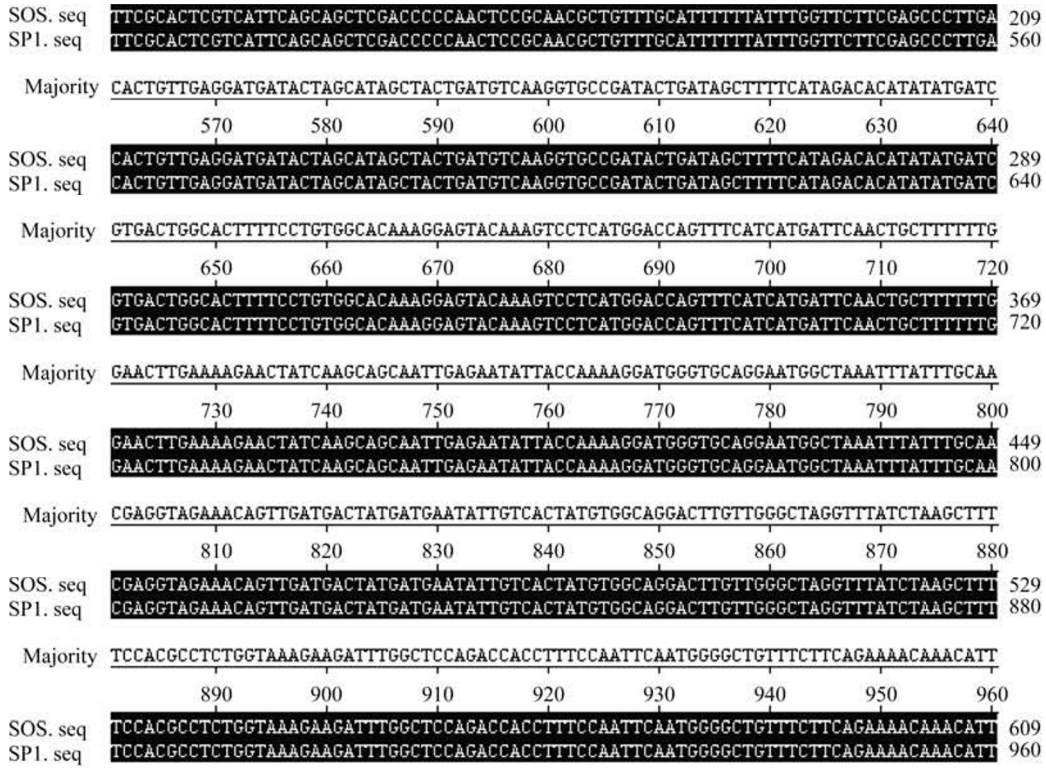

Fig. 3 Sequencing results of recombinant plasmid pPIC-*Gu*SQS1

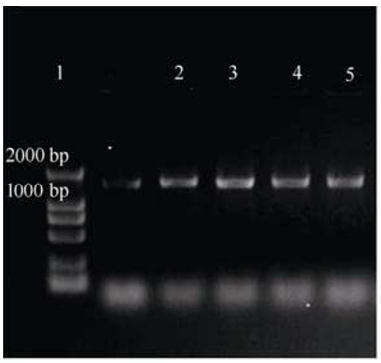

Fig. 4 PCR results of recombinant *P. pastoris* with *Gu*SQS1 gene Lane 1 is the marker; lanes 2−5 are fragments obtained by PCR from recombinant *P. pastoris* with *Gu*SQS1 gene

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 *Gu*SQS1 was finally fixed to be 24, according to the results of preliminary experiments. RT-PCR analysis showed that *Gu*SQS1 expressed in alltransgenic *P. pastoris* at different levels (Fig. 5). As shown in Fig. 6, the relative expression of *Gu*SQS1 in recombinant *P. pastoris* containing 4-copy-*Gu*SQS was higher than that in the rest of recombinant *P. pastori*s 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 *Gu*SQS1 in different recombinant *P. pastoris*

No. of strains	Average Ct value		Y value in standard curve		Copy numbers of *Gu*SQS1 (*Gu*SQS1/*GAP*)
	GAP	*Gu*SQS1	*GAP*	*Gu*SQS1	
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	1.1×10^5	1.8×10^6	16
6	26.35	20.98	9.5×10^2	4.6×10^4	47

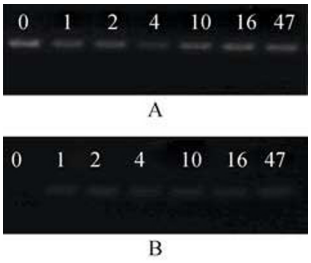

Fig. 5 Semi-quantitative RT-PCR results in different recombinant *P. pastoris* A represents RT-PCR results of *GAP*; B represents RT-PCR results of *Gu*SQS1; and the numbers on the picture represent the copy number of *Gu*SQS1 in the recombinant *P. pastoris*

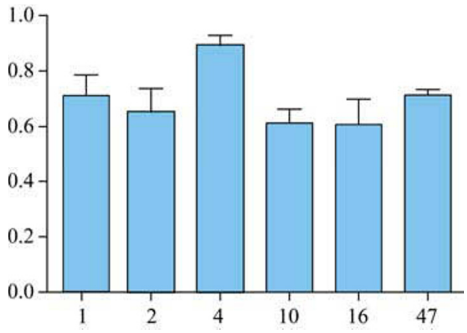

Fig. 6 The expression of *Gu*SQS1 in different recombinant *P. pastoris* strains

standard curve for ergosterol was as follows: $Y = 3.818\ 4 \times 10^{-5}X - 7.930\ 2 \times 10^{-3}$ ($R^2 = 0.999\ 9$). And it had good linearity between 0.1 μg·mL^{-1} to 50.0 μg·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 analsys results with independent-sample T test results (n = 3) are listed in Table 3. The contents of ergosterol in all recombinant *P. pastoris* containing *Gu*SQS1 were 1.15−1.96 times higher than that in the negative control. And with the increase in the copy number of *Gu*SQS1, 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

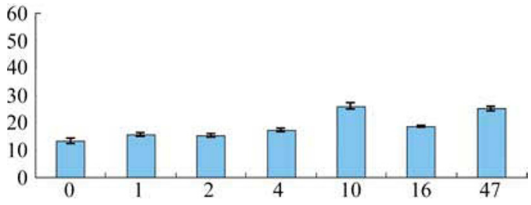

Fig. 7 The contents of ergosterol in different recombinant *P. pastoris* (X-axis: the copy number of *Gu*SQS1 in different recombinant *P. pastoris*, Y-axis: the contents of ergosterol (μg))

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 *Gu*SQS1 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 substantial 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 *Gu*HMGR 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 [35-38]. 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 *Gu*SQS1. Using real-time PCR, the copy numbers of *Gu*SQS1 in different transgenic *P. pastoris* were 1, 2, 4, 10, 16, and 47 copies, respectively. The RT-PCR results showed that *Gu*SQS1 was expressed in transgenic *P. pastoris* at different levels. And the relative expression of *Gu*SQS1 in recombinant *P. pastoris* containing 4-copy-*Gu*SQS1 was higher than that in other recombinant *P. pastori*s. Because *SQS*1 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 *Gu*SQS1 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 *Gu*SQS1 the content of ergosterol presented in an increasing-decreasing-increasing pattern. In the recombinant *P. pastoris* containing 10 copies and 47 copies of *Gu*SQS1 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 *Gu*SQS1 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 *Gu*SQS1 was higher than that in recombinant *P. pastoris* containing 1 copy, 2 copies and 4 copies of *Gu*SQS1. 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 *Gu*SQS1 in recombinant *P. pastoris* containing 16-copy- *Gu*SQS1 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 [39-40]. So it is possible that increasing of squalene synthase and 2, 3-oxidosquale 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 *Gu*SQS1 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 *Gu*SQS1 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 *Gu*SQS1 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 *Gu*SQS1 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* [9] also harbor glycyrrhizin. Therefore, this study could form basis for further studies on glycyrrhizin biosynthesis in those medicinal plants.

Abbreviations

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
SQS, squalene synthase

TCM, traditional Chinese medicine
YPD, yeast peptone dextrose medium

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References

[1] Zeng L, Li SH, Lou ZC. Morphological and histological studies of Chinese licorice [J]. *Acta Pharm Sin*, 1988, **23**: 200-208.

[2] State Pharmacopoeia Committee. *Pharmacopoeia of China, Part I* [M]. Beijing: Chemical Industry Press, 2010: 80-81.

[3] Cherng JM, Lin HJ, Hsu YH, *et al*. A quantitative bioassay for HIV-1 gene expression based on UV activation effect of glycyrrhizic acid [J]. *Antiviral Res*, 2004, **62**(1): 27-36.

[4] Hoever G, Bzltina L, Michaelis M. Antiviral activity of glycyrrhizic acid derivatives against SARS-coronavirus [J]. *J Med Chem*, 2005, **48**(4): 1256-1259.

[5] Hyung KK, Yoonkyung P, Hee NK. Antimicrobial mechanism of b-glycyrrhetinic acid isolated from licorice. *Glycyrrhiza glabra* [J]. *Biotechnol Lett*, 2002, **24**(22): 1899-1902.

[6] Shibata S. A drug over the millennia: Pharmacognosy, chemistry, and pharmacology of licorice [J]. *Yakugaku Zasshi*, 2000, **120** (10): 849-862.

[7] Van Rossum TG, Vulto AG. Intravenous glycyrrhizin for the treatment of chronic hepatitis C: a double blind, randomized, placebo-controlled phase I/II trial [J]. *J Gastroenterol Hepatol*, 1999, **14**(11): 1093-1099.

[8] Wei SL, Wang WQ, Wang JY, *et al*. Preliminary study in glycyrrhizin content and its influencing factors of wild and cultivated in different region of China [J]. *China J Chin Mat Med*, 2012,**37**(10): 1341-1345.

[9] Hayashi H, Fukui H, Tabata M. Examination of triterpenoids produced by callus and cell suspension cultures of *Glycyrrhiza glabra* [J]. *Plant Cell Rep*, 1988, **7**(7): 508-511.

[10] Hayashi H, Huang P, Inoue K. Up-regulation of soyasaponin biosynthesis by methyl Jasmonate in cultured cells of *Glycyrrhiza glabra* [J]. *Plant Cell Physiol*, 2003, **44**(4): 404-411.

[11] Hayashi H, Sakai T, Fukui H. Formation of soyasaponins in licorice cell suspension cultures [J]. *Phytochemistry*, 1990, **29**(10): 3127-3129.

[12] Ayabe S, Takano H, Fujita T, *et al*. Triterpenois biosynthesis in tissue cultures of *Glycyrrhiza glaba* var. *glanduliera* [J]. *Plant Cell Rep*, 1990, **9**(4): 181-184.

[13] Paulet LC, Cairo A, Pavia PB, *et al*. Enhanced flux through the methylerythritol 4-phosphate pathway in *Arabidopsis* plants overexpressing deoxyxylulose 5-phosphate reductoisomerase [J]. *Plant Mol Biol*, 2006, **62**(4-5): 683-695.

[14] Zhang L, Yang B, Lu BB, *et al*. Tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures over-expressing *Putrescine N*-methyltransferase is methyl jasmonate-dependent [J]. *Planta*, 2007, **225**(4): 887-896.

[15] Liu Y, Zhang N, Chen HH, *et al*. Cloning and characterization of two cDNA sequences coding squalene synthase involved in glycyrrhizic acid biosynthesis in *Glycyrrhiza uralensis* [J]. *Lect Notes Electric Engineer*, 2013, **269**: 329-342.

[16] Liu DJ. *Researches on the correlation between CNVs of HMGR, SQS1, β-AS gene and origin and morphology of Glycyrrhiza uralensis* [D]. Dissertation, Beijing University of Chinese Medicine, 2011: 8-11.

[17] Lu HY, Liu JM, Zhang HC, *et al*. Ri-mediated transformation of *Glycyrrhiza uralensis* with a squalene synthase gene (GuSQS1) for production of glycyrrhizin [J]. *Plant Mol Biol Rep*, 2008, **26**(1): 1-11.

[18] Lee MH, Jeong JH, Seo JW, *et al*. Enhanced triterpene and phytosterol biosynthesis in Panax ginseng overexpressing squalene synthase gene [J]. *Plant Cell Physiol*, 2004, **45**(8): 976-984.

[19] Seo JW, Jeong JH, Shin CG, *et al*. Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation [J]. *Phytochemistry*, 2005, **66**(8): 869-877.

[20] Mirjalili MH, Moyano E, Bonfill M, *et al*. Overexpression of the *Arabidopsis thaliana* squalene synthase gene in *Withania coagulans* hairy root cultures [J]. *Biologia plantarum*, 2011, **55**(2): 357-360.

[21] Kribii R, Arro M, Arco AD, *et al*. Cloning and characterization of the *Arabidopsis thaliana* SQS1 gene encoding squalene synthase involvement of the C-terminal region of the enzyme in the channeling of squalene through the sterol pathway [J]. *Eur J Biochem*, 1997, **249**(1): 61-69.

[22] Hayashi H, Huang P, Inoue K. Up-regulation of soyasaponin biosynthesis by methyl Jasmonate in cultured cells of *Glycyrrhiza glabra* [J]. *Plant Cell Physiol* , 2003, **44**(4): 404-411.

[23] Uchida H, Yamashita H, Kajikawa M, *et al*. Cloning and charaterization of a squalene synthase gene from a petroleum plant, *Euphorbia tirucalli* L. [J]. *Planta*, 2009, **229**(6): 1243-1252.

[24] Lee JH, Yoon YH, Kim HY, *et al*. Cloning and expression of squalene synthase cDNA from hot pepper (*Capsicum annuum* L.) [J]. *Mol Cells*, 2002, **13**(3): 436-443.

[25] Zhang HC, Liu JM, Lu HY, *et al*. Enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* Fisch by combining the over-expression of chalcone isomerase gene with the elicitation treatment [J]. *Plant Cell Rep*, 2009, **28**(8): 1205-1213.

[26] Kang TW, Jeon YJ, Jang E, *et al*. Copy number variations (CNVs) identified in Korean Individuals [J]. *BMC Genomics*, 2008, **9**(1): 492-499.

[27] Palta P, Kaplinski L, Nagirnaja L, *et al*. Haplotype phasing and inheritance of copy number variants in nuclear families [J]. *PLoS One*, 2015, **10**(4): e0122713.

[28] Redon R, Ishikawa S, Fitch KR, *et al*. Global variation in copy number in the human genome [J]. *Nature*, 2006, **444**(7118):

444-454.

[29] Beckmann JS, Estivill X, Antonarakis SE. Copy number variants and genetic traits: closer to the resolution of phenotypic to genotypic variability [J]. *Nat Rev Genet*, 2007, **8**(8): 639-646.

[30] Stranger BE, Forrest MS, Dunning M, *et al*. Relative impact of nucleotide and copy number variation on gene expression phenotypes [J]. *Science*, 2007, **315**(5813): 848-853.

[31] Hastings PJ, Lupski JR, Rosenberg SM, *et al*. Mechanisms of change in gene copy number [J]. *Nat Rev Genet*, 2009, **10**(8): 551-564.

[32] Ausubel FM, Kinston RE, Seidman JG, *et al*. *Short Protocols in Molecular Biology* [M]. 4th ed. Beijing: Science Press; 2005, p. 25.

[33] Zhu XQ. *Researches on the influences of CNVs of functional genes HMGR, SQS and β-AS on their expression in Glycyrrhiza uralensis* [D]. Beijing University of Chinese Medicine, 2012: 40-41.

[34] Waterham HR, Digan ME, Koutz PJ, *et al*. Isolation of the Pichia Pastoris glyceradehyde-3-phosphate dehydrosgenasee gene and regulation and use of its promoter [J]. *Gene*, 1997, **186**(1): 37-44.

[35] Liu Y, Liu DJ, Liu CS, *et al*. Mechanism of genuineness of liquorice *Glycyrrhiza uralensis* based on CNVs of HMGR, SQS1 and *β*-AS gene [J]. *Acta Pharm Sin*, 2012, **47**(2): 250-255.

[36] Liu Y, Xu QX, Wang XY, *et al*. Researches on the influence of 3-hydroxy-3-methylglutary-coenzyme A reductase gene polymorphism on catalytic efficiency of its encode enzyme in *Glycyrrhiza uralensis* [J]. *China J Chin Mat Med*, 2012, **37**(24): 3784-3788.

[37] Liu Y, Xu QX, Wang XY, *et al*. Analysis on correlation between 3-hydroxy-3-methylglutary-coenzyme A reductase gene polymorphism of *Glycyrrhiza uralensis* and content of glycyrrhizic acid [J]. *China J Chin Mat Med*, 2012, **37**(24): 3789-3792.

[38] Liu Y, Zhang N, Wang XY, *et al*. Researches on influence of squalene synthase gene polymorphism on catalytic efficiency of its encode enzyme in *Glycyrrhiza uralensis* [J]. *China J Chin Mat Med*, 2012, **37**(24): 3777-3783.

[39] Larkin PJ, Miller JAC, Allen RS, *et al*. Increasing morphinan alkaloid production by over-expressing codeinone reductase in transgenic *Papaver somniferum* [J]. *Plant Biotechnol J*, 2007, **5**(1): 26-37.

[40] White PJ. The regulation of K^+ influx into roots of rye (*secale cereale* L.) seedlings by negative feedback *via* the K^+ flux from shoot to root in the phloem [J]. *J Exp Bot*, 1997, **48**(12): 2063-2073.

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