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ORIGINAL ARTICLE

Enhancing production of ergosterol in *Pichia pastoris* GS115 by over-expression of 3-hydroxy-3-methylglutaryl CoA reductase from *Glycyrrhiza uralensis*

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KEY WORDS

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Over-expression;
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Copy number variation

Abstract The rate-limiting enzyme in the mevalonic acid (MVA) pathway which can lead to triterpenoid saponin glycyrrhizic acid (GA) is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). In order to reveal the effect of copy number variation in the HMGR gene on the MVA pathway, the HMGR gene from *Glycyrrhiza uralensis* Fisch. (*Gu*HMGR) was cloned and over-expressed in *Pichia pastoris* GS115. Six recombinant *P. pastoris* strains containing different copy numbers of the *Gu*HMGR gene were obtained and the content of ergosterol was analyzed by HPLC. The results showed that all the recombinant *P. pastoris* strains contained more ergosterol than the negative control and the strains with 8 and 44 copies contained significantly more ergosterol than the other strains. However, as the copy number increased, the content of ergosterol showed an increasing–decreasing–increasing pattern. This study provides a rationale for increasing the content of GA through over-expressing the *Gu*HMGR gene in cultivars of *G. uralensis*.

Abbreviations: BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; CNV, copy number variation; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LOD, limit of detection; LLOQ, lower limit of quantitation; MD, minimal dextrose medium; MM, minimal medium; MVA, mevalonic acid; PCR, polymerase chain reaction; RSD, relative standard deviation; YPD, yeast peptone dextrose medium

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1. Introduction

Besides the use as an industrial raw material and tobacco additive, the roots of *Glycyrrhiza uralensis* Fisch. are widely used in many Chinese herbal remedies for their ability to nourish "Qi", alleviate pain, tonify the spleen and stomach, eliminate phlegm and relieve cough[1,2]. The source of this pharmacological activity is a number of active components of which glycyrrhizic acid (GA) is considered the most important. This has led to its adoption as a marker compound of the quality of *G. uralensis*. Many studies have shown that GA possesses antiinflammatory, antitumor and immune-stimulating activities[3–7].

Excessive exploitation of wild *G. uralensis* plants in the years leading up to 2000 decreased the supply to such an extent that the Chinese government imposed restrictions on their collection. As a result, cultivars have now become the main source of this herb. However, the low content of GA in these cultivars has placed severe restrictions on their sustainable development. Attempts have been made to solve this problem using cell suspensions of *G. uralensis*[8–11] but without success. We therefore decided to genetically engineer *G. uralensis* plants through modifying the triterpene biosynthetic pathway which leads to the formation of GA.

In the biosynthesis of GA, the rate-limiting enzyme is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR)[12–16] which catalyzes the reaction of HMG-CoA and NADPH to form mevalonic acid (MVA). Many previous studies[17–19] have shown that the accumulation of terpenes is significantly increased by increasing the content of the HMGR gene but, to date, over-expression of the HMGR gene in *G. uralensis* (*Gu*HMGR) to increase the production of GA has not been reported. In this study, we investigated how copy number variation (CNV) of the *Gu*HMGR gene affects the formation of ergosterol. We maintain that the results indicate that over-expression of the *Gu*HMGR gene increases the accumulation of GA in cultivars of *G. uralensis*.

2. Materials and methods

2.1. Construction of the yeast expression vector containing GuHMGR gene

*Not*I and *Sna*BI of pPIC9K (Fig. 1) were selected as the specific enzyme cutting sites to insert the *Gu*HMGR gene. Primer pairs with the specific enzyme sites underlined are as follows:

HF: 5′-CGGTACGTAATGGACGTTC GCCGGAG-3′ (*Sna*BI)
HR: 5′-ATAGCGGCCGCTGGAGGCTT TCGTTATTGGT-3′ (*Not*I)

The cycling parameters of PCR were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, annealing at 64 °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 purified and subcloned into pMD19-T (Takara, Japan). The resulting vector (*Gu*HMGR-T) was digested with *Sna*BI (2 h at 37 °C) and *Not*I (2 h at 37 °C) and then subcloned into pPIC9K (Invitrogen, USA). The resulting recombinant pPIC9K–*Gu*HMGR plasmid was transferred into the disarmed *E. coli* DH5α[20] and sequenced for correct insertion.

2.2. Construction of recombinant P. pastoris containing GuHMGR gene

The recombinant pPIC9K–*Gu*HMGR plasmid was linearized by restriction enzyme *Sal*I and mobilized by electroporation (1500 V, 25 μF, 400 Ω) into the disarmed *P. pastoris* GS115 (Invitrogen, USA). An aliquot (0.5 mL) of yeast peptone dextrose (YPD) medium was then added and the cells were cultured at 30 °C, 200 rpm for 1 h. An aliquot (200 μL) of the suspension was placed on minimal dextrose (MD) solid medium and cultured at 30 °C for 2 days. Single colonies were removed and incubated on minimal medium (MM) and MD solid medium simultaneously at 30 °C for 2–4 days; the colonies growing on both MM and MD media were selected.

PCR was used to check that the recombinant *P. pastoris* contained the *Gu*HMGR gene. The single colonies were used as PCR template[21] and primers were as follows: forward primer, 5′-TACTATTGCCAGCATTGCTGC-3′; reverse primer, 5′-GCAA ATGGCATTCTGACATCC-3′. The 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.

Selected recombinant *P. pastoris* was induced to express the *Gu*HMGR gene using BMGY and BMMY liquid media (30 °C, 250 rpm). The supernatant from a 96 h culture was examined by 12% SDS-PAGE using Coomassie brilliant blue staining. *P. pastoris* containing a void vector was used as a negative control.

2.3. Copy number determination

The GAP gene was selected as the internal control gene for real-time PCR[22]. The primer pair of GAP (GenBank accession number: U62648) was as follows: GF: 5′-CACAATGGCTATCACTGTCG-3′; GR: 5′-GACACACTACAGCCCGCATT-3′. The primer pair of the *Gu*HMGR gene was as previously stated. The 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 transformed into disarmed *E. coli* DH5*α*. Then the standard plasmids pMD19-T–*Gu*HMGR and pMD19-T–GAP were obtained, extracted and diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 copy numbers/2 μL. For real-time PCR analysis, the primer pairs in Table 1 were used with the following cycling parameters: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s, saving at 4 °C. Standard curves of C_t (Cycle threshold) on the *X*-axis and log (concentration of standard plasmid) on the *Y*-axis were constructed. All recombinant *P. pastoris* strains were amplified by real-time PCR. The ratio of the *Y* values of *Gu*HMGR and GAP was taken as the copy number of the *Gu*HMGR gene in each recombinant *P. pastoris* strain.

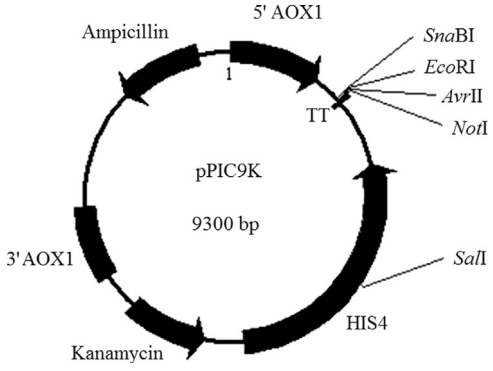

Figure 1 Structure of pPIC9K.

Table 1 Primers used for real-time PCR analysis.

Gene	Primer
GAP	rGF: 5′-GGTATTAACGGTTTCGGACGTATTG-3′ rGR: 5′-GATGGTGACAGGGTCTCTCTCTTGG-3′
GuHMGR	rHF: 5′-CACGGTTTCCTCGTCTTCAA-3′ rHR: 5′-CGTCTACCTCCTCGGCTTCTT-3′

2.4. Semi-quantitative RT-PCR analysis

Total RNA was isolated from different recombinant *P. pastoris* strains using a yeast RNA rapid extraction kit (Beijing BoMaiDe Medical Technology Co., Ltd.). To remove plasmid DNA, RNase-free DNase I enzyme (Tiangen Biotech Co., Ltd.) was used according to the manufacturer's instructions. Spectrophotometry was used to determine the concentration of RNA. The cycle number was set at 18, 20, 22, 24 and 26, and the optimal cycle number determined by electrophoresis in 1% (*w/v*) agarose gel. The cycling parameters of RT-PCR were as follows: 50 °C for 30 min; 94 °C for 2 min; optimal cycles of 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s; and a final extension at 72 °C for 10 min; saving at 4 °C. The primer pair, GF (5′-CACAATGGCTATCACTGTCG-3′) and GR (5′-GACACTACAGCCCGCATT-3′), was used to amplify the GAP gene as an internal control.

2.5. Assay of ergosterol in recombinant P. pastoris

HPLC analysis of ergosterol in samples was carried out on a Waters 2695 system equipped with a Phenomenex LUNA C18 column (250 mm × 4.6 mm, 5 μm) using a mobile phase of methanol:water 97:3 (*v/v*) delivered at a flow rate of 1.0 mL/min. The detection wavelength was 283 nm and the injection volume 20 μL.

A stock solution of ergosterol (purity: 97.7%) containing 11.38 mg in 10 mL absolute ethanol was used to prepare a series of standard solutions containing 0.01%, 0.05%, 0.1%, 0.5%, 2%, and 5% of the stock solution in absolute ethanol. Intra-day precision (as relative standard deviation, RSD) was determined by replicate analysis ($n=6$) of a solution containing 5.559 μg/mL ergosterol. The limit of detection (LOD) and lower limit of quantity (LLOQ) were determined using sequentially more dilute solutions of ergosterol. Recovery was assessed using 9 samples of blank *P. pastoris* cells accurately weighed (50 mg) and spiked with 2.91 μg, 5.05 μg and 8.41 μg ergosterol.

Recombinant *P. pastoris* strains containing different copy numbers of the *Gu*HMGR gene were induced to express the gene; *P. pastoris* GS115 without the *Gu*HMGR gene was used as negative control. All 96 h cultures were collected by centrifugation at 5000 rpm and lyophilized. Samples of the dried powders (50 mg) were extracted into 8 mL ethyl acetate by ultrasonication for 1 h. The ethyl acetate was evaporated to dryness, the residue reconstituted in 2 mL ethanol analyzed for ergosterol.

3. Results

3.1. Construction of the yeast expression vector containing the GuHMGR gene

A 1745 bp fragment was shown by PCR and BLAST analysis to have a 99% identical sequence to that of the *Gu*HMGR gene (GenBank accession number: GQ345405.1). It was successfully inserted at the *Sna*BI–*Not*I site of pPIC9K to give the recombinant plasmid pPIC–*Gu*HMGR shown in Fig. 2 where lane 1 is the marker and lanes 2 and 3 are fragments obtained by PCR with the correct length.

3.2. Construction of recombinant P. pastoris containing GuHMGR gene

The linearized pPIC–*Gu*HMGR was transformed to *P. pastoris* GS115. Most single colonies of recombinant *P. pastoris* simultaneously growing on MM and MD media were shown by PCR to have the correct fragment length. After inducing for 96 h, the negative control was dark yellow while the recombinant *P. pastoris* was yellowish-white. SDS-PAGE (Fig. 3) showed a band between 86 and 47 kDa in samples from recombinant *P. pastoris* which was not present in the negative control. These results demonstrate that the construction and inducible expression of recombinant *P. pastoris* strains containing the *Gu*HMGR gene were successful.

3.3. Copy number determination of the GuHMGR gene in transgenic P. pastoris

PCR and sequencing showed that the standard plasmids were correct. The melting curves of the GAP and *Gu*HMGR genes were both unimodal suggesting that the primers used in real time PCR were specific. Using real time PCR, two fragments with 220 and 237 bp were obtained which sequencing and BLAST analysis showed were the *Gu*HMGR gene and the GAP gene of *P. pastoris*, respectively. Their standard curves were described by the equations $Y=-2.609X+32.21$ ($R^2=0.995$) and $Y=-2.994X+35.27$ ($R^2=0.997$), correspondingly. The copy numbers of the *Gu*HMGR gene in the different transgenic *P. pastoris* strains were found to be 1, 2, 4, 8, 13 and 44 (Table 2).

3.4. Semi-quantitative RT-PCR analysis of transgenic P. pastoris

Semi-quantitative RT-PCR was employed to detect the relative abundance of the *Gu*HMGR gene in the transgenic *P. pastoris* strains. The cycle number was finally fixed at 20 based on a

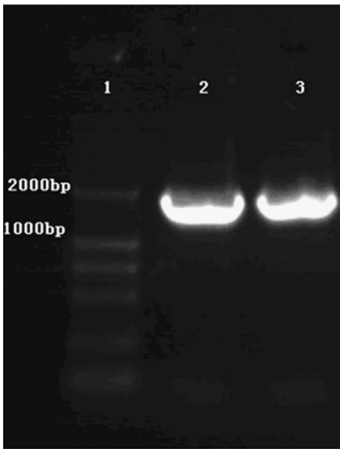

Figure 2 PCR analysis of the construction of recombinant plasmid pPIC–*Gu*HMGR. Lane 1: marker; lanes 2 and 3: fragments obtained by PCR.

preliminary experiment. RT-PCR analysis revealed the *Gu*HMGR gene was expressed in all transgenic *P. pastoris* strains at different levels (Fig. 4b) whereas expression of the GAP gene was roughly similar (Fig. 4a). As shown in Fig. 4c, the relative expression of the *Gu*HMGR gene in the recombinant *P. pastoris* strain containing 4 copies was higher than in the other strains consistent with the results listed in Table 3 ($P<0.05$ for the strain containing 4 copies versus all other strains).

3.5. *Assay of ergosterol in recombinant P. pastoris*

The retention time of ergosterol in HPLC was 8.16 min. The standard curve was linear over the concentration range 0.1–50.0 μg/mL and described by the equation $Y=3.8184\times10^{-5}X-7.9302\times10^{-3}$ ($R^2=0.9999$). The LLOQ (*S/N* of 10) was 2.22 ng and the LOD (*S/N* of 3) 0.89 ng. The assay was precise (RSD 0.0592%, $n=6$) with recovery in the range 97.3%–101.0% (RSD 0.55%–0.82%). The content of ergosterol in all samples is shown in Fig. 5. The results of independent *t*-tests of the content of ergosterol in different recombinant *P. pastoris* strains ($n=3$) are listed in Table 4. The level of ergosterol in all recombinant *P. pastoris* strains was 1.07–2.51 times higher than in the negative control but with increase in the copy number of *Gu*HMGR gene; the content of ergosterol showed an increasing–decreasing–increasing pattern. For strains with copy number <4, the content of ergosterol was similar; at copy number 8, the content of ergosterol was highest (2.5 times the negative control); for copy number 13, the content of ergosterol was only 1.04 times negative control; and for copy number 44, the content of ergosterol was 1.8 times negative control. Clearly, the copy number of the *Gu*HMGR gene influences the level of ergosterol in transgenic *P. pastoris*.

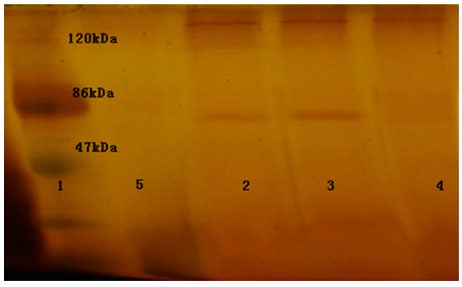

Figure 3 SDS-PAGE analysis of the expression of the *Gu*HMGR gene. Lane 1: marker; lanes 2–4: recombinant *P. pastoris* containing the *Gu*HMGR gene; lane 5: negative control.

4. Discussion

In our previous studies, we found functional genes in *G. uralensis* such as those of HMGR and SQS were subject to CNV[23–25]. We were therefore interested to analyze the relationship between the

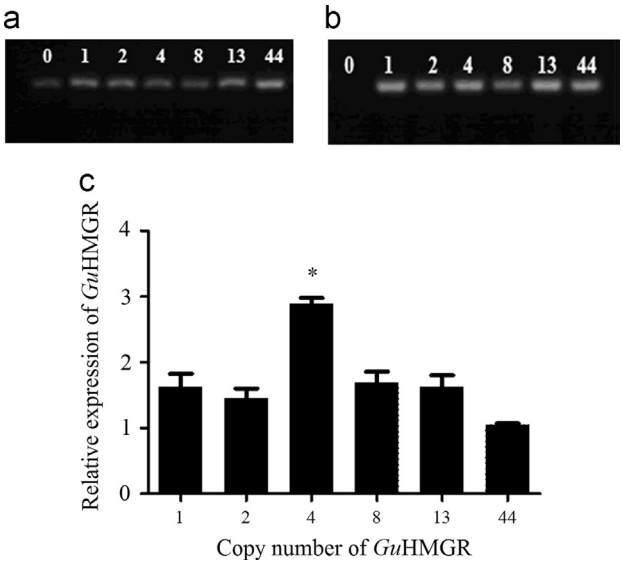

Figure 4 Semi-quantitative RT-PCR analysis of the expression of the *Gu*HMGR gene in different recombinant *P. pastoris* strains. (a) RT-PCR results of the GAP gene; (b) RT-PCR results of the *Gu*HMGR gene (the numbers are the copy numbers of the *Gu*HMGR gene in the recombinant *P. pastoris* strains); (c) relative expression of the *Gu*HMGR gene in recombinant *P. pastoris* strains with different copy numbers.

Table 3 Independent *t*-test results of expression of the *Gu*HMGR gene in different recombinant *P. pastoris* strains by RT-PCR.

Copy number	*P* value					
	1	2	4	8	13	44
1	–	0.425	0.000	0.736	0.994	0.019
2	0.425	–	0.000	0.264	0.421	0.082
4	0.000	0.000	–	0.000	0.000	0.000
8	0.736	0.264	0.000	–	0.741	0.010
13	0.994	0.421	0.000	0.741	–	0.018
44	0.019	0.082	0.000	0.010	0.018	–

Table 2 Copy numbers of the *Gu*HMGR gene in different recombinant *P. pastoris*.

No. of strain	Average C_t value		*Y* value in standard curve		Copy number of *Gu*HMGR (*Gu*HMGR/*GAP*)
	GAP	*Gu*HMGR	*GAP*	*Gu*HMGR	
1	23.10	21.18	1.2×10^4	1.7×10^4	1
2	17.29	15.51	1.0×10^6	2.5×10^6	2
3	24.26	20.83	4.7×10^3	2.3×10^4	4
4	20.38	16.81	9.4×10^4	8.0×10^5	8
5	22.36	18.06	2.0×10^4	2.7×10^5	13
6	25.12	19.07	2.5×10^3	1.1×10^5	44

copy number of functional genes and the content of GA acid in *G. uralensis*.

CNV arises from deletions, insertions, duplications, and more complex variations ranging from 1 kb to submicroscopic sizes[26]. Genes with CNV have wide distribution, hereditability, relative stability and high heterogeneity. CNV can lead to changes in gene dosage and phenotypic character and, to date, many reports have documented a close relationship between CNV and human disease[27–30]. Despite the high level of interest in CNV, there are few reports of CNV in plant genes.

In this study, recombinant *P. pastoris* strains containing 1, 2, 4, 8, 13 and 44 copies of the *Gu*HMGR gene were constructed. RT-PCR analysis revealed that the *Gu*HMGR gene was expressed in all transgenic *P. pastoris* strains at different levels with the strain containing 8 copies, showing highest expression as indicated by the content of ergosterol. However, with increasing copy number, the content of ergosterol did not increase in a linear fashion but showed an increasing–decreasing–increasing pattern with the strains containing 8 and 44 copies containing higher levels of ergosterol than those containing 1, 2, 4 and 13 copies.

The reason for the non-linear dependence of expression on copy number is unclear but one possibility is that it involves gene silencing caused by integration sites of exogenous genes. Recently, several studies have demonstrated feedback inhibition in over-expressing exogenous genes in plants[31,32] and it may be that an increase in the *Gu*HMGR gene results in feedback inhibition of upstream steps of the MVA pathway to reduce the accumulation of ergosterol. Then when an enzyme level decreases, feedback inhibition may be interrupted leading to reopening of the metabolic pathway which could explain why the level of ergosterol is increased in the *P. pastoris* strain containing 44 copies of the *Gu*HMGR gene.

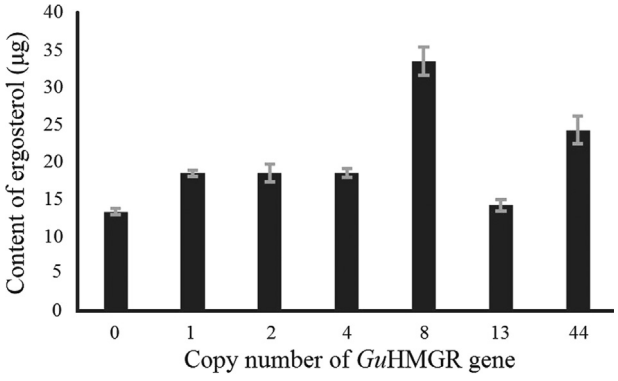

Figure 5 Content of ergosterol in recombinant *P. pastoris* strains with different copy numbers of the *Gu*HMGR gene ($n=3$).

Table 4 Independent *t*-test results of the content of ergosterol in different recombinant *P. pastoris* strains ($n=3$).

Copy number	*P* value						
	0	1	2	4	8	13	44
0	–	0.000	0.002	0.000	0.000	0.142	0.001
1	0.000	–	0.959	0.847	0.000	0.001	0.006
2	0.002	0.959	–	0.951	0.000	0.006	0.011
4	0.000	0.847	0.951	–	0.000	0.001	0.007
8	0.000	0.000	0.000	0.000	–	0.000	0.004
13	0.142	0.001	0.006	0.001	0.000	–	0.001
44	0.001	0.006	0.011	0.007	0.004	0.001	–

In this study, the dependence of the content of ergosterol on the copy number of the *Gu*HMGR gene suggests that an increase in the latter could lead to an increase in the production of GA in *G. uralensis*. However, it must be recognized that the production of GA involves a very complex metabolic network which is regulated and controlled by many key enzymes, of which HMGR is but one. Nevertheless we maintain that the current results provide an important basis for further studies aimed at increasing the GA content of *G. uralensis* and exploring its biosynthesis *in vitro*. In addition, other herbs used in Chinese medicine such as *Glycyrrhiza glabra* and *Glycyrrhiza inflate* also produce GA and this work is relevant to further studies of its biosynthesis in these medicinal plants.

Acknowledgments

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