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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

Glycyrrhiza uralensis Fisch.;
3-Hydroxy-3-methylglutaryl-CoA reductase gene;
Over-expression;
Pichia pastoris;
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.

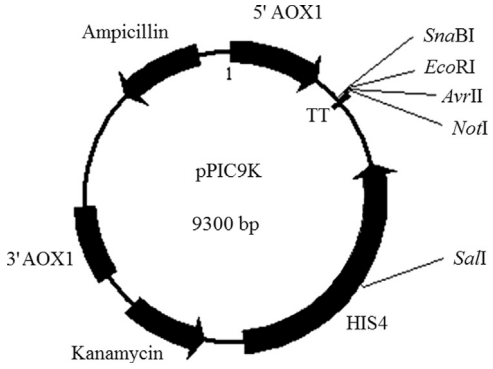

Figure 1 Structure of pPIC9K.