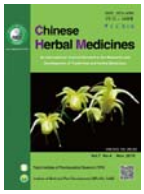

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Review

HMGR, *SQS*, *β-AS*, and Cytochrome P450 Monooxygenase Genes in *Glycyrrhiza uralensis*

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ABSTRACT

Glycyrrhiza uralensis is frequently used in traditional Chinese medicine. This plant contains a large amount of effective constituents, including triterpenoids and flavonoids. Among them, glycyrrhizin is believed to be the marker compound to evaluate the quality of *G. uralensis* based on *Chinese Pharmacopoeia*. Many studies showed that glycyrrhizin possesses various pharmacological activities, such as antibacterial, antiviral, antitumor, anti-inflammatory, and immune-stimulating activities. In this paper, we summarized the cloning, characterization, expression, and polymorphism analysis of several functional genes involved in glycyrrhizin biosynthesis in *G. uralensis*.

Key words

β-AS; cytochrome P450 monooxygenase; functional genes; glycyrrhizin; HMGR; SQS

1. Introduction

Glycyrrhiza uralensis Fisch. (*Gancao* in Chinese) is widely used in Chinese herbal compound prescriptions (Zeng et al, 1988; Pharmacopoeia Committee of P. R. China, 2010). Among its various natural active components, glycyrrhizin, one of the triterpenoids derived from the roots and rhizomes of *G. uralensis*, is believed to be the major bioactive compound to characterize the quality of this Chinese herb based on *Chinese Pharmacopoeia*. Many studies have reported that glycyrrhizin possesses various biological activities, such as antibacterial (Long et al, 2013; Yoshida et al, 2010), antiviral (Huang et al, 2012; Wang et al, 2013; Baltinar et al, 2012), antitumor (Sun et al, 2010; Park et al, 2009; Zhang et al, 2011), anti-inflammatory (Wu et al, 2011; Chandrasekaran et al, 2011), renoprotective (Yu et al, 2010) and immune-stimulating activities (Kim et al, 2013; Li et al, 2012). Although the pharmacological activities of glycyrrhizin have been extensively researched, its biosynthesis remains poorly understood. Up to date, it is commonly believed that glycyrrhizin is biosynthesized through mevalonic acid (MVA) pathway, which is controlled and regulated by many enzymes (Figure 1).

In recent years, the reports about functional genes involved in glycyrrhizin biosynthesis concentrated mainly on the following genes, 3-hydroxy-3-methyliuglutary CoA reductase (HMGR) gene, which encodes the first rate-limiting enzyme involved in MVA pathway, squalene synthase (SQS) gene and beta-amyrin synthase (*β*-AS) gene, which encode enzymes playing the important roles in the early stages of triterpene skeleton formation, and cytochrome P450 mono-oxygenase genes, which encode enzymes involved in the multiple oxidations at positions C-11 and C-30, and glycosylation

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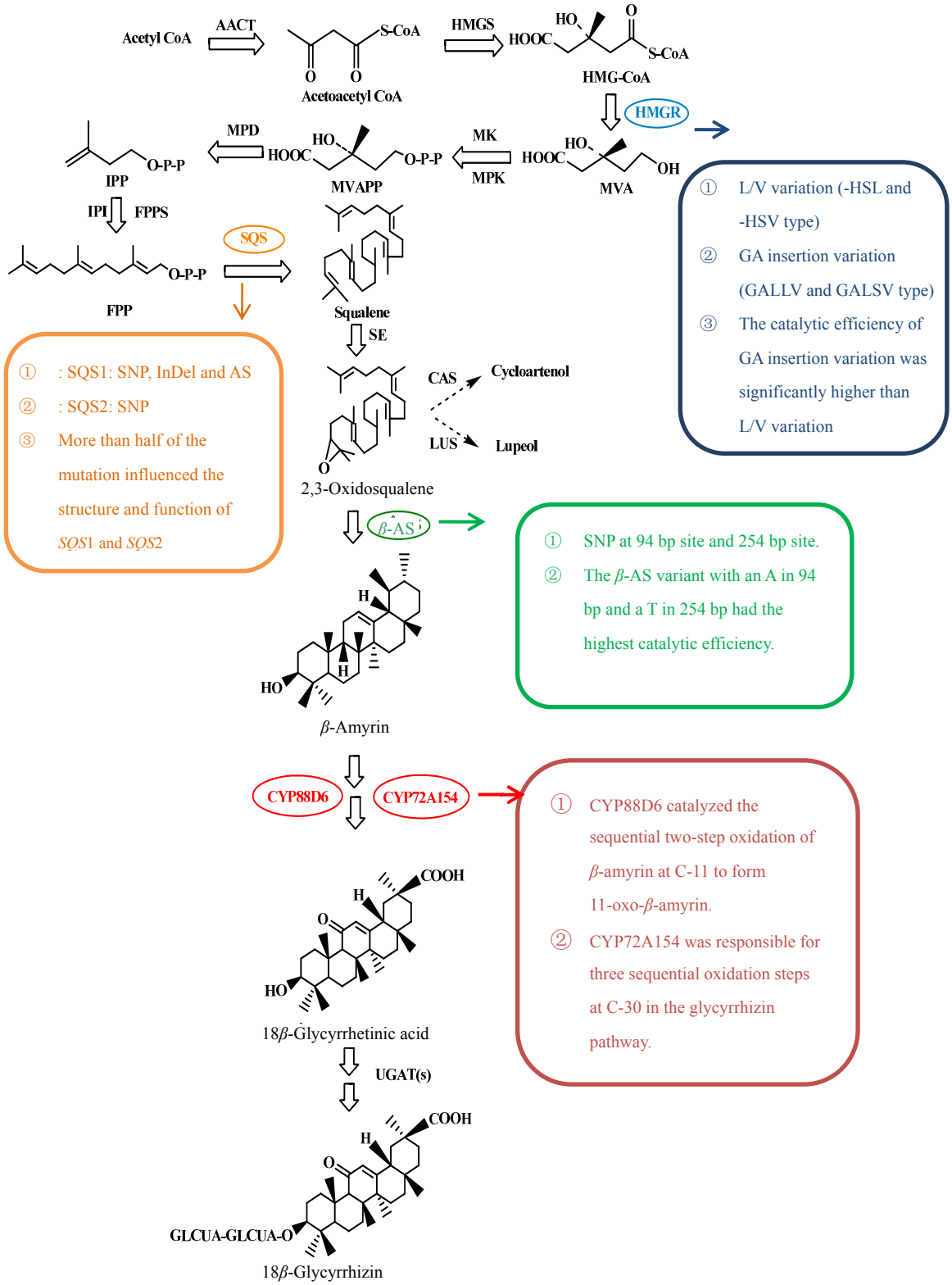

Figure 1 Biosynthetic pathway of glycyrrhizin

AACT: acetyl-CoA C-acetyltransferase; HMGS: hydroxymethylglutaryl-CoA synthase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; MK: mevalonate kinase; MPK: mevalonate phosphate kinase; MVA: mevalonate; MVAPP: mevalonate pyrophosphate; MPD: mevalonate pyrophosphate decarboxylase; IPP: isopentenyl pyrophosphate; IPI: isopentenyl pyrophosphate isomerase; FPPS: farnesyl pyrophosphate synthase; FPP: farnesyl pyrophosphate; SQS: squalene synthase; SE: squalene epoxidase; CAS: cycloartenol synthase; LUS: lupeol synthase

of the C-3 hydroxyl group leading from *β*-amyrin to glycyrrhizin. In this paper, the cloning, characterization, expression, and polymorphism analysis of the above functional genes involved in glycyrrhizin biosynthesis were summarized combining with our own experimental experiences. And we hope this work can provide a basis for further studies concerned with exploring the biosynthesis of glycyrrhizin *in vitro* and strengthening the efficacy of *G. uralensis* by means of improved glycyrrhizin content.

2. *HMGR* gene

HMGR is believed to be the first rate-limiting enzyme involved in glycyrrhizin biosynthesis (Harker et al, 2003; Aquil et al, 2009; Friesen and Rodwell, 2004; Yang et al, 1991). It catalyzes HMG-CoA and NADPH into MVA, which is an irreversible reaction. Many reports (Chappell and Nable. 1987; Schaller et al, 1995; Dai et al, 2001) have shown that the accumulation of terpene increased significantly with the increasing of the content of *HMGR* gene.

In our previous studies, rapid-amplification of cDNA ends (RACE) was used to clone the cDNA of *HMGR* from *G. uralensis*. A 1842 bp full-length cDNA sequence (GenBank accession number: GQ845405) was obtained, including an ORF of 1722 bp (between 39 and 1760 bp) that encoded a 573-residue protein, a 38 bp 5′-UTR and an 82 bp 3′-UTR. It was subcloned into disarmed vector, introduced to *E. coli* BL21 cells and expressed. SDS-PAGE analysis demonstrated the appearance of a 6×10^4 Da recombinant protein, which was absent from the equivalent fractions of negative control. In the following enzymatic reaction, both TLC and GC-MS analyses demonstrated that a new product, mevalonolactone (MVL) was produced, while it was not present in the negative control (Liu et al, 2013a).

In the further studies, the polymorphisms of *HMGR* gene sequence and amino acid sequence in *G. uralensis* were analyzed (Liu et al, 2012a; 2012b). It was found that single nucleotide polymorphism (SNP), insertion deletion length polymorphism (InDel) and heterozygosity were present in *HMGR* gene sequence. The SNPs appeared at 17 different sites, including 72, 81, 99, 185, 432, 465, 500, 544, 669, 685, 792, 900, 1008, 1089, 1389, 1509, and 1709 bp, and the insertion of GTGGCG appeared between 74−79 bp. The heterozygosity of G/A, C/T, A/G, and T/C was determined at 432, 465, 792, and 1089 bp, separately. In its amino acid sequence there were two kinds of variation, L/V variation (-HSL and -HSV) and GA insertion variation (GALLV and GALSV). The amino acid sequences of type -HSL were mutated to histidine (H), serine (S), and leucine (L), and the amino acid sequences of type -HSV were mutated to H, S, and valine (V) at 62, 167, and 229 amino acid residue sites, respectively. In type GALLV, glycine (G) and alanine (A) were inserted at the 25−26 sites, in addition, the 62, 167, and 229 sites were mutated to L, L, and V. While in the type GALSV, amino acid was mutated to S at 167 sites, and other mutation sites were same compared with type GALLV. -HSL mutant was only present in group with a low content of glycyrrhizin, while GALSV mutant was only present in group with a high content of glycyrrhizin. After a catalytic efficiency test, it showed that the catalytic efficiency of GA insertion variation was significantly higher than L/V variation. This conclusion was in accord with the content of glycyrrhizin in *G. uralensis*.

3. *SQS* gene

SQS is situated in a branch point from farnesyl diphosphate (FPP) to triterpenoids or other products in the MVA pathway and may be an up-regulator for triterpene skeleton formation (Lu et al, 2008; Lee et al, 2004; Seo et al, 2005). *SQS* gene belongs to a multigene family with two kinds of *SQS* gene found in *Arabidopsis thaliana* (Mirjalili et al, 2011; Kribii et al, 1997), *Glycyrrhiza glabra* (Hayashi et al, 2003), and several other plants.

Two cDNA sequences, *SQS*1 and *SQS*2, have been cloned from *G. uralensis* (Liu et al, 2013b). A 1241 bp full-length cDNA sequence (GenBank accession number: AM182329) of *SQS*1 gene encoding a 413-residue protein and a 1239 bp full-length cDNA sequence (GenBank accession number: AM182330) of *SQS*2 gene encoding a 412-residue protein were obtained respectively. These two *SQS* gene sequences cloned from *G. uralensis* have a 98% similarity with the two *SQS* gene sequences cloned from *G. glabra* by Hayashi (Hayashi et al, 2003). They were subcloned and transformed into *E. coli* BL21 competent cells and expressed. SDS-PAGE analysis demonstrated that the presence of a 6.7×10^4 Da recombinant protein, which was absent from the negative control. In the following enzymatic reaction, both TLC and GC-MS analyses demonstrated that a new product, squalene, was produced, while it was not present in the negative control (Liu et al, 2012c).

In the further studies, the polymorphism of *G. uralensis SQS* gene sequence and amino acid sequence were analyzed. It showed that SNP, InDel, nonsense mutation, and alternative splicing (AS) were present in *SQS*1 gene sequence. There were many SNPs in *SQS*1 gene cDNA sequences, including A/G transition at 98, 250, 425, 426, 689, 922, and 984 bp sites, C/T transition at 23, 385, 495, 502, 1052, 1117, 1118, and 1231 bp sites, C/G transversion at 1121 bp site, T/G transversion at 606 bp site, A/T transversion at 314, 374, and 1159 bp sites, A/G transversion at 98 and 718 bp sites, and A/G transversion at 63, 520, and 898 bp sites. In a few *SQS*1 gene cDNA sequences, 3 basic groups (GGA) were deleted at 738–740 bp sites, which resulted in the deletion of one amino acid residue. The AS in *SQS*1 gene cDNA sequences included intron retention (a 15 bp fragment inserted between 1036–1051 bp) and exon skipping (a 76 bp fragment deleted between 329–404 bp). While for *SQS*2 gene sequences, there was only one kind of polymorphism, SNP, including C/T transition at 396, 513, 660, and 1210 bp sites, C/G transition at 741 bp site, and A/T transversion at 1177 bp site. In the amino acid sequence of SQS1, the substitutions of 17 amino

acids were found at the sites of 8, 33, 84, 105, 125, 129, 142, 146, 168, 230, 240, 308, 328, 351, 373, 374, and 387. The conservative substitution was 47.06%, and non-conservative substitution was 52.94%, which demonstrated that more than half of the mutation influenced the structure and function of SQS1. In the amino acid sequence of SQS2, the substitutions of five amino acid residues were found at the sites of 174, 247, 330, 393, and 404. The conservative substitution was 40%, and non-conservative substitution was 60%, which also demonstrated that more than half of the mutation influenced the structure and function of SQS2. The catalytic efficiency of different SQS protein was quite different.

4. *β-AS* gene

β-AS is situated in a branch point to catalyze 2, 3-oxidosqualene into *β*-amyrin, which also plays an important role for triterpene skeleton formation (Hayashi et al, 2011; Wang et al, 2011).

A 2289 bp full-length cDNA sequence (GenBank accession number: FJ627179) of *β-AS* encoding a 762-residue protein was obtained from *G. uralensis*. In the further study (Chen et al, 2013) a 4109 bp full-length DNA sequence of *β-AS* was obtained from *G. uralensis*. Compared to the *β-AS* cDNA sequence, the 14 extrons and 13 introns were determined. The *β-AS* gene was subcloned and introduced into *Saccharomyces cerevisiae* competent cells and expressed. SDS-PAGE analysis demonstrated the appearance of an 8.7×10^4 Da recombinant protein, which was absent from the negative control. In the following enzymatic reaction, both TLC and GC-MS analyses demonstrated that a new product, *β*-amyrin, was produced, while it was not present in the negative control, which proved that the *β-AS* gene we obtained had a normal catalytic activity.

In the further studies, the polymorphism of *β-AS* gene sequence and amino acid sequence was analyzed. It was found that SNP in 94 bp site was a missense mutation, and SNP in 254 bp site was a same sense mutation. The catalytic efficiency of different *β*-amyrin synthase encoded by different *β-AS* variants was obviously different. The *β-AS* variant with an A in 94 bp and a T in 254 bp had the highest catalytic efficiency.

The temporal and spatial specificity of *β-AS* gene expression in *G. uralensis* had also been studied (Liu and Liu, 2012). The spatial specificity experiment showed that *β-AS* did not express in the above ground part of *G. uralensis*, while in the underground part, the expression of root tip was higher than rootstock. This conclusion is accord with the distribution of glycyrrhizin content level in *G. uralensis*. And the temporal specificity experiment showed that the expression of *G. uralensis β-AS* could be divided into four stages. From December to February, the expression of *β-AS* was lower than the detection level; from March to May, *β-AS* began to express; from May to September, the expression of *β-AS* kept a high level; and in October and November, the expression of *β-AS* began to decrease.

5. Cytochrome P450 monooxygenase gene

Hikaru et al reported the cytochrome P450 mono-oxygenase involved in glycyrrhizin biosynthesis (Hikaru et al, 2008; 2011). Two cytochrome P450 monooxygenase genes, CYP88D6 and CYP72A154, have been successfully identified. *In vitro* enzymatic activity assays showed that CYP88D6 catalyzed the sequential two-step oxidation of *β*-amyrin at C-11 to form 11-oxo-*β*-amyrin, which was a possible biosynthetic intermediate between *β*-amyrin and glycyrrhizin. CYP72A154 was responsible for three sequential oxidation steps at C-30 in the glycyrrhizin pathway. Both CYP88D6 and CYP72A154 expressed in the roots only, but not in the leaves or stems, which is consistent with the accumulation pattern of glycyrrhizin in plant. Two additional CYP72A subfamily members were also identified, CYP72A153 (60.0% identity with CYP72A154) and CYP72A155 (50.9% identity with CYP72A154), however, neither of them showed detectable activity.

6. Discussion

G. uralensis is one of the most widely used Chinese herbs for its effects of nourishing *qi*, tonifying spleen and stomach, relieving coughing and eliminating phlegm. The market demand for *G. uralensis* is very huge in China. However, due to recent years' excessive consumption, wild resources of *G. uralensis* are becoming endangered. The Chinese government has imposed the restrictions on the collection of wild *G. uralensis* plants. As a result, cultivation has become the main source of *G. uralensis* supply. However, the degradation of quality and low content of glycyrrhizin are widely present in the cultivar of *G. uralensis*. Consequently improving the quality of cultivar becomes the key issue of the sustainable development. Through modifying the triterpen biosynthesis pathway which leads to the formation of glycyrrhizin, it is possible to find an effective approach to increase the accumulation of glycyrrhizin in *G. uralensis* plants (Jin et al, 2010).

Up to date, the metabolic pathway of glycyrrhizin shows that many enzymes play essential roles in this procedure. Based on the central dogma, functional gene polymorphisms may result in amino acid polymorphisms, and ultimately affect the enzyme catalytic efficiency. The abundant gene polymorphisms of *HMGR*, *SQS*, and *β-AS* may influence the catalytic functions of their encoded enzymes. It has been found that the catalytic efficiency of GA insertion variation was significantly higher than L/V variation. More than half of the mutation influenced the structure and function of *SQS*1 and *SQS*2 gene. The *β-AS* variant with an A in 94 bp and a T in 254 bp had the highest catalytic efficiency. It is known that the tiny mutation of functional gene possibly influence the encoding enzyme in quality and quantity. Therefore, the clear understanding of functional genes involved in glycyrrhizin biosynthesis is very significant for revealing the molecular mechanism of glycyrrhizin formation. We hope this work can provide a basis for improving the quality of *G. uralensis* cultivars.

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