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Analysis of functional gene polymorphism in *Glycyrrhiza uralensis*

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***Glycyrrhiza uralensis* is widely used in Chinese herbal compound prescriptions. It contains various natural active components, among them glycyrrhizic acid is believed to be the main effective constituent. The biosynthetic pathway of glycyrrhizic acid is controlled and regulated by many different enzymes. In our studies two functional genes coding 3-hydroxy-3-methylglutary CoA reductase (HMGR) and squalene synthase (SQS) were selected as target genes. Several kinds of polymorphism were discovered in the cDNA sequences and amino acid sequences of the two genes, and which inflected the level of glycyrrhizic acid in *G. uralensis*.**

Key words: *Glycyrrhiza uralensis*; HMGR, SQS, gene polymorphism, glycyrrhizic acid

Glycyrrhiza uralensis Fisch. (Gancao in Chinese) is widely used in Chinese herbal compound prescriptions. Based on traditional Chinese medicine theory, *G. uralensis* was believed to have the functions of nourishing *qi*, relieving coughing, alleviating pain, tonifying spleen and eliminating phlegm (Zeng et al. 1988, State Pharmacopoeia Committee 2010). The reason why *G. uralensis* has these potent effects and wide applications is that it contains lots of natural active components, including more than 20 categories of triterpenoids and approximately 300 categories of flavonoids. Many modern clinic studies have reported that *G. uralensis* possesses various biological activities, such as anti-bacterial (Ai-Turki et al. 2008, Park et al. 2008, Bodet et al. 2008), anti-inflammatory (Cheng et al. 2008, Sun and Pan 2006, Wu et al. 2011), anti-tumor (Tatsuzaki et al. 2007, Park et al. 2009, Lee et al. 2008) and antiviral activities (Romero et al. 2005, Sasaki et al. 2002-2003, Cinatl et al. 2003). Besides the medicinal value, *G. uralensis* is also used as food corrective agents and tobacco additives in China.

The very large demand of *G. uralensis* has caused the irresponsible excessive exploitation of wild *G. uralensis* resources. In 2000, the Chinese government had to impose restrictions on the collection of wild *G. uralensis*. As a result cultivars have become the main resource of this herb in Chinese herbal medicine markets. However, the content of glycyrrhizic acid in most of cultivars can not accord with the minimum standard (2%) in Chinese pharmacopoeia. Consequently improving the quality of cultivars of *G. uralensis* has become the key issue of the sustainable development. We think it is interesting and meaningful to solve this problem by genetic engineering.

Among all the active components in *G.*

uralensis, glycyrrhizic acid is believed to be the main effective constituent and treated as an indicator component to characterize the quality of this Chinese herb. Up to now the biosynthetic pathway of glycyrrhizic acid has been clarified (Fig. 1), which is controlled and regulated by many enzymes, among them two enzymes (marked in red in Fig. 1) are very important. 3-hydroxy-3-methylglutary CoA reductase (HMGR) is the first rate-limiting enzyme in this metabolic pathway, it catalyzes HMG-CoA and NADPH into MVA, which is an irreversible reaction (Harker et al. 2003, Aquil et al. 2009). Squalene synthase (SQS) lies in the branch point from FPP to triterpenoids or other products, and when positively regulated it can help the synthesis of triterpenoids (Lu et al. 2008). Therefore in our present studies the cDNAs coding the above two enzymes were cloned and the polymorphism of them were observed. It was also found that the functional gene polymorphism inflected the level of glycyrrhizic acid. Gene variations can cause the differences in enzyme activity and expression and finally lead to the differences in level of metabolites. The influence of functional gene polymorphism on metabolic pathway is especially significant, which has been studied in the field of medical science and drug screening (Ozaydin et al. 2010, Sugimoto et al. 2007, Deng et al. 2011). However, reports about the influence of functional gene polymorphism on the accumulation of glycyrrhizic acid in *G. uralensis* are very infrequent. We hope our studies can do some help to reveal the relation between functional genes and the content of glycyrrhizic acid.

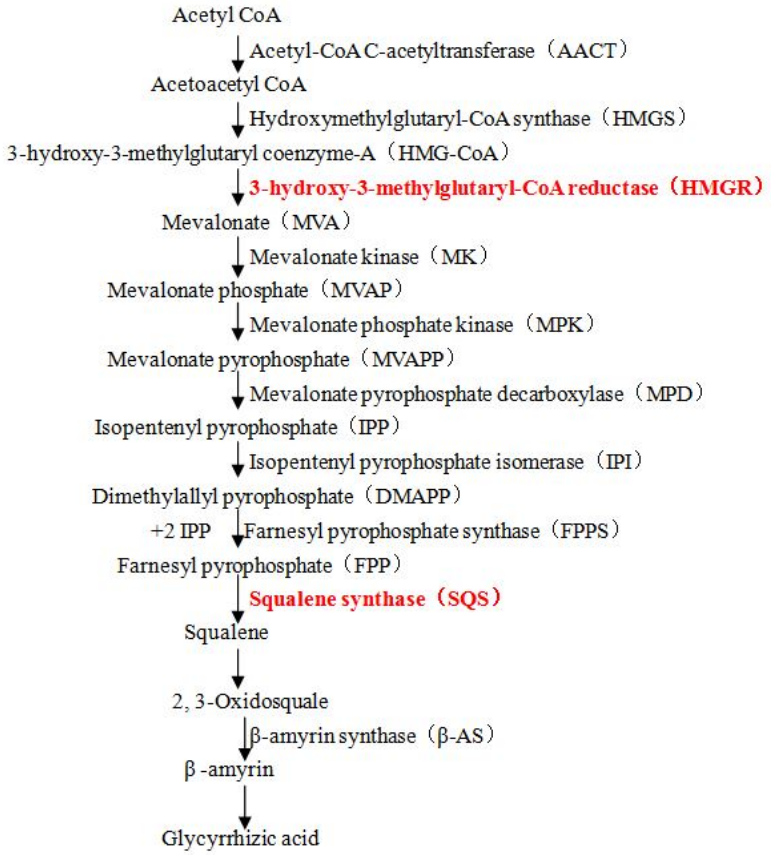

Fig. 1 Biosynthetic pathway of glycyrrhizic acid

Materials and methods

Plant material and RNA extraction

The roots of *G. uralensis* were collected from the herb garden in Beijing University of Chinese Medicine, Beijing, China. Samples were immersed in liquid nitrogen during the field collection and identified by Prof. Liu Chun-sheng (Beijing University of Chinese Medicine). Total RNA was extracted from approximately 1g fresh tissue using the "Trizol" reagent (Beijing MeiLaiBo medical technology Co., LTD), following the manufacturer's instructions. Final RNA concentrations were determined by spectrophotometry and their integrity was examined by electrophoresis in 1% (w/v) agarose gel.

Molecular cloning of HMGR cDNA from *G. uralensis*

Single-stranded cDNA was synthesized from 12μL of total RNA using the primers oligo (dT) and reverse transcriptase M-MLV (Takara) in 50μL reactions, following the manufacturer's instructions. After aligning HMGR sequences of *Leguminous* plant recorded in Genbank (accession number: XM003612517.1 and GI357484554), the amplification step was performed using primers HMGR-F1 (5'-GGG TCG GAA AAT GGA CGT-3') and HMGR-R1 (5'-GGA GGC TTT CGT TAT TGG TC-3') designed using conserved regions. The cycling parameters were as follows: 94°C for 5min; 35cycles of 94°C for 30sec, annealing at 57°C for 30sec, extension at 72°C for 2min; a final extension at 72°C for 7min. The amplified fragments were subcloned into pMD19-T, and the recombinant plasmids HMGR-T were obtained, which were transformed into disarmed *E.coli* DH5α cells, and DNA sequencing was carried out by Shanghai Sangon biological technology co., LTD.

Molecular cloning of SQS cDNA from *G. uralensis*

Based on the SQS cDNA sequences recorded in Genbank (AM182329 and D86409), the primers were designed using conserved regions (Table 1). The cycling parameters were as follows: 94°C for 5min; 35cycles of 94°C for 30sec, annealing at 57°C for 30sec, extension at 72°C for 90sec; a final extension at 72°C for 10min. The amplified fragments were subcloned into pMD19-T, and the recombinant plasmid SQS1-T and SQS2-T were obtained, which were transformed into disarmed *E.coli* DH5α cells, and DNA sequencing was carried out by Shanghai Sangon biological technology co., LTD.

Table 1 Primer pairs used in this paper

Gene	Primer	Sequence (5'-3')
SQS1	SQS-1F	ATGGGGAGTTTGGGAGCGAT
	SQS-1R	CGTGTTTGACCATTCGTTTC
SQS2	SQS-2F	ATGGGGAGTTTGGGAGCGAT
	SQS-2R	CTAGTTATTTTGGCGGTTGGCAG

Bioinformatics analysis of HMGR and SQS in *G. uralensis*

The obtained HMGR sequences and SQS sequences were aligned using BLAST on the website NCBI, ORF Finder was used to look for the open reading frame. DNAclub was used to analyze the polymorphism of amino acid sequences.

Results and discussion

Polymorphism of HMGR gene in *G. uralensis*

Twelve different cDNA sequences (No. 1~ No. 12) coding 3-hydroxy-3-methylglutary CoA reductase were obtained from different *G. uralensis* plants. The mutations in the twelve HMGR cDNA sequences were listed in Table 2. Based on Table 2, it was determined that single nucleotide polymorphism (SNPs) appeared in 17

different sites, and insertion deletion length polymorphism (InDel) appeared between 74 ~ 79 bp. The mutations in the relevant amino acid sequences were listed in Table 3. It was determined that missense mutation appeared in three different sites (62, 167, 229), deletion and insertion mutation appeared between 25th ~ 26th residue. The following enzymatic reaction experiments showed that the insertion mutation (glycine and alanine in 25 ~ 26 site) was beneficial to the accumulation of glycyrrhizic acid.

Table 2 The mutations in different HMGR cDNA sequences of *G. uralensis*

Sequences	Mutation sites (bp)								
	72	74-79	81	99	185	432	465	500	544
1	C	-	C	G	T	G	T	C	T
2	C	-	G	A	A	G	C	C	C
3	C	-	G	A	A	A	T	C	C
4	C	-	G	A	A	G	C	C	C
5	C	-	G	A	A	G	C	C	C
6	C	CTGGCG	G	A	A	G	C	C	C
7	C	CTGGCG	C	G	T	G	T	T	C
8	T	-	C	A	A	A	T	C	C
9	C		G	G	A	A	T	C	C
10	C	CTGGCG	C	G	T	G	T	C	C
11	T	-	C	G	A	A	T	C	C
12	C	-	G	A	A	G/A	C/T	C	C
Sequences	Mutation sites (bp)								
	669	685	792	900	1008	1089	1389	1509	1709
1	G	G	A	G	G	T	T	G	T
2	A	T	A	A	G	T	A	A	C
3	A	G	G	G	G	C	T	A	C
4	A	G	A	G	G	T	A	A	T
5	A	T	A	G	G	T	A	A	T
6	A	T	A	G	G	T	A	A	C
7	G	G	G	G	A	T	A	T	T
8	A	G	G	G	G	C	T	A	T
9	A	G	G	G	G	C	T	A	T
10	G	G	G	G	G	T	A	A	T
11	A	G	G	G	G	C	T	A	T
12	A	G	A/G	G	G	T/C	A	A	T

Table 3 Mutations in different HMGR amino acid sequences of *G. uralensis*

Sequences	Mutation sites (amino acid residues)			
	25-26	62	167	229
1	deletion	His (H)	Ser (S)	Leu (L)
2	deletion	His (H)	Ser (S)	Leu (L)
3	deletion	His (H)	Ser (S)	Val (V)
4	deletion	His (H)	Ser (S)	Val (V)
5	deletion	His (H)	Ser (S)	Leu (L)
6	Glycine (G) alanine (A)	Leu (L)	Leu (L)	Val (V)
7	Glycine (G) alanine (A)	Leu (L)	Ser (S)	Val (V)
8	deletion	His (H)	Ser (S)	Val (V)
9	deletion	His (H)	Ser (S)	Val (V)
10	Glycine (G) alanine (A)	Leu (L)	Leu (L)	Val (V)
11	deletion	His (H)	Ser (S)	Val (V)
12	deletion	His (H)	Ser (S)	Val (V)

Polymorphism of SQS gene in *G. uralensis*
SQS gene belongs to a multigene family, up to now two kinds of SQS genes with different expressing activities have been found in many plants, such as *Arabidopsis thaliana* (Mirjalili et al, 2011, Kribii et al. 1997) and *Glycyrrhiza glabra* (Hiroaki et al. 2003).In our studies twelve different SQS1 cDNA sequences and five different SQS2 cDNA sequences were obtained. For SQS1 cDNA sequences, comparing with the conservative sequence (Genbank: HM012825) there were four kinds of polymorphism, including SNPs, InDel, nonsense mutation and alternative splicing (AS). SNPs appeared in 22 different sites (Table 4), the ratio of missense mutation was about 80.9%. InDel appeared between 738 ~ 740 bp, 3 basic groups (GGA) was missing, which led to the deletion of one amino acid residue. Nonsense mutation appeared in 1231 bp, C mutated into T. In one SQS1 cDNA sequence, a 15 bp fragment was inserted between 1036 ~ 1051 bp, and in another SQS1 cDNA sequence, a 76 bp fragment was deleted between 329 ~ 404 bp (Fig 2). These polymorphism both belonged to AS, the former was intron retention and the latter was exon skipping. For SQS2 cDNA sequences, there was only one kind of polymorphism, SNPs (Table 4). It appeared in 9 different sites, and the ratio of missense mutation was about 55.5%.

Table 4 SNPs in SQS1 and SQS2 cDNA sequences of *G. uralensis*

	Mutation types	Mutation sites (bp)
SQS1	A/G transition	98, 250, 425, 436, 689, 922, 984
	C/T transition	23, 385, 495, 502, 1052, 1117, 1118, 1231
	C/G transversion	1121
	T/G transversion	606
	A/T transversion	314, 374, 1159
	A/C transversion	98, 718
SQS2	A/G transition	63, 520, 898
	C/T transition	396, 513, 660, 1210
	C/G transition	741
	A/T transversion	1177

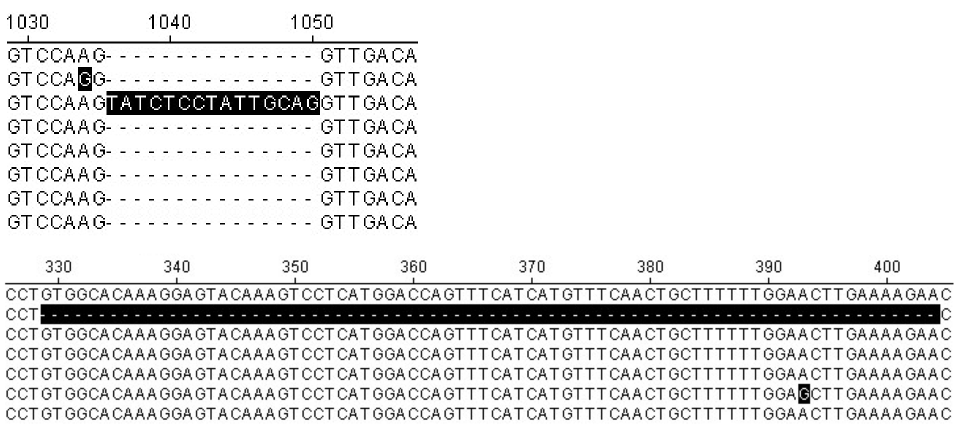

Fig. 2 Alternative splicing appeared in SQS1 cDNA sequences of *G. uralensis*

All the cDNA sequences were translated into amino acid sequences by DNAclub, 17 different mutations were discovered in the amino acid sequences of SQS1 (Table 5) and 5 different mutations were discovered in the amino acid sequences of SQS2 (Table 6). In the following enzymatic reactions it was found that the catalytic efficiency of different recombinant SQS1 protein was different,

which was influenced by conservative substitution obviously, but the catalytic efficiency of different recombinant SQS2 protein was similar to each other, which declared that the polymorphism of SQS2 could not change the enzyme activity.

Table 5 The mutations in SQS1amino acid sequences of *G. uralensis*

Mutation sites (amino acid residue)	Before the mutation	After the mutation	type
8	Val (V)	Ala (A)	Conservative substitution
33	Ala (A)	Glu (E)/Gly (G)	Non-conservative substitution
84	Ser (S)	Gly (G)	Non-conservative substitution
105	Val (V)	Asp (D)	Non-conservative substitution
125	Val (V)	Asp (D)	Non-conservative substitution
129	Phe (F)	Leu (L)	Conservative substitution
142	Asn (N)	Ser (S)	Conservative substitution
146	Arg (R)	Gly (G)	Non-conservative substitution
168	Tyr (Y)	His (H)	Non-conservative substitution
230	Arg (R)	Gln (Q)	Non-conservative substitution
240	Leu (L)	Ile (I)	Conservative substitution
308	Arg (R)	Gly (G)	Non-conservative substitution
328	Met (M)	Ile (I)	Conservative substitution
351	Pro (P)	Leu (L)	Conservative substitution
373	Leu (L)	Pro (P)/Ser (S)	Non-conservative substitution
374	Ser (S)	Thr (T)	Conservative substitution
387	Asn (N)	Tyr (Y)	Conservative substitution

Table 6 The mutations in SQS2 amino acid sequences of *G. uralensis*

Mutation sites (amino acid residue)	Before the mutation	After the mutation	type
174	Gly (G)	Arg (R)	Non-conservative substitution
247	Asp (D)	Glu (E)	Conservative substitution
330	Asp (D)	Gly (G)	Non-conservative substitution
393	Phe (F)	Ile (I)	Conservative substitution
404	Tyr (Y)	His (H)	Non-conservative substitution

It is well known that the metabolic pathway of glycyrrhizic acid is very complex, which is regulated and controlled by many enzymes. HMGR and SQS are only two of them. We just hope our work can provide a basis for further studies concerned with exploring the biosynthesis of glycyrrhizic acid *in vitro* and strengthening the efficacy of *G. uralensis* by improving the content of glycyrrhizic acid. There are still lots of interesting work waiting us to do.

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