Pak. J. Bot., 50(1): 73-84, 2018.

ENHANCING GLYCYRRHIZIC ACID ACCUMULATION BY ROOT-SPECIFICALLY OVER-EXPRESSING 3-HYDROXY-3-METHYLGLUTARY COA REDUCTASE (HMGR), SQUALENE SYNTHASE1 (SQS1), AND *B*-AMYRIN SYNTHASE (*B*-AS) GENES IN *GLYCYRRHIZA URALENSIS*

BO-CHUAN YUAN[1,a], RUI YANG[1,a], YONG-SHENG MA[1], SHAN ZHOU[1], WEN-DONG LI[2] AND YING LIU[1,*]

[1]School of Chinese Pharmacy, Beijing University of Chinese Medicine
[2]Department of Antibiotic, Beijing Institute for Drug Control
[*]Correspondence author's email: liuyliwd@sina.com Phone: +861084738646 Fax: +861084738611
[a]Contributed equally authors: These authors contributed equally to this paper

Abstract

Glycyrrhizia uralensis is one of the most widely-used Chinese herbal medicines, and glycyrrhizic acid (GA) is its marker component. However, the GA content in *G. uralensis* cultivars is generally low. We selected three functional genes involved in GA biosynthetic pathway in *G. uralensis*, 3-hydroxy-3-methylglutary CoA reductase gene (*GuHMGR*), squalene synthase1 gene (*GuSQS*1), and *β*-amyrin synthase gene (*GuBAS*), and constructed three root-specific expression vectors by gene fusion, then transformed them into disarmed *Agrobacterium tumefaciens* EHA105 cells using $CaCl_2$ freeze-thaw method, which was used to infect *G. uralensis* explants. There were about 13% explants survived in infection and then dedifferentiated to resistant callus. The shoots differentiation rate was only about 7%, but they all successfully took root and finally, about 60% regenerated *G. uralensis* plantlets survived in transplantation. qRT-PCR was used to analyze the expression level and calculate the copy number of *GuHMGR*, *GuSQS*1, and *GuBAS* genes in the regenerated transgenic *G. uralensis* plants. Root-specific over-expression of *GuHMGR*, *GuSQS*1, and *GuBAS* genes were respectively observed in the regenerated transgenic *G. uralensis* plants. Thirteen *GuHMGR* transgenic *G. uralensis* plants were obtained with the gene copy number of 2, 3, 4, 5, or 6, respectively, six *GuSQS*1 transgenic *G. uralensis* plants were obtained with the gene copy number of 2, 3, 4, or 6, respectively, and five *GuBAS* transgenic *G. uralensis* plants were obtained with the gene copy number of 3 and 5, respectively. HPLC was used to detect the contents of GA and isoglycyrrhizic acid (IGA) in regenerated *G. uralensis* plants. It was found that GA and IGA contents were both higher in transgenic plants than that in blank control, and with the increase of copy number of *GuHMGR*, *GuSQS*1, and *GuBAS* both contents were increased. Root-specific over-expression of *GuHMGR*, *GuSQS*1, and *GuBAS* genes enhances the accumulation of GA and IGA.

Key words: *Glycyrrhiza uralensis*; *HMGR*; *SQS*; *β-AS*; Root-specific; Over-expression.

Abbreviations:
β-AS = *β*-amyrin synthase, Cef = cefotaxim sodium, CNVs = copy number variations, FP = forward primer, FPP = farnesyl diphosphate, GA = glycyrrhizic acid, HMGR = 3-hydroxy-3-methylglutary CoA reductase, HPLC = high-performance liquid chromatography, Hyg = hygromycin, IGA = isoglycyrrhizic acid, Kan = kanamycin sulfate, LB = Luria-Bertani, MS = Murashige and Skoog, MVA = mevalonic acid, OD = optical density, qRT-PCR = quantitative real-time PCR, RP = reverse primer, SQS = squalene synthase, TCM = traditional Chinese medicine, YEB = Yeast Extract Broth

Introduction

With a 3000-year medicinal history, *Glycyrrhiza uralensis* is believed to be one of the most important herbal medicines in China (Wei *et al.,* 2012). The roots and rhizomes of *G. uralensis* are widely used in traditional Chinese medicine (TCM) for their functions of relieving coughing, nourishing *qi*, tonifying spleen and stomach, alleviating pain and eliminating phlegm (Anon., 2010). *G. uralensis* is also frequently used as industrial raw materials and tobacco additives. Glycyrrhizic acid (GA), a marker of *G. uralensis*, has been studied for its antitumor (Chueh *et al.,* 2012; Khan *et al.,* 2013), anti-inflammatory (Bhattacharjee *et al.,* 2012; Luo *et al.,* 2013), antiviral (Matsumoto *et al.,* 2013; Smirnov *et al.,* 2012), antidiabetic (Alqahtani *et al.,* 2013; Feng *et al.,* 2013), hepatoprotective activities (Rasool *et al.,* 2014; Tripathi *et al.,* 2009), and many other activities (Kratschmar *et al.,* 2011; Ma *et al.,* 2013; Raikkonen *et al.,* 2010; Cynthia *et al.,* 2011). Therefore, research about the medicinal uses of GA have been attracting increasing attention.

However, with the increase of demand, wild *G. uralensis* resources have shown a progressive decline. Chinese government has to impose a strict restriction on the collection and commercial uses of wild *G. uralensis*, which leads to the cultivars becoming the main source of *G. uralensis* medicinal materials. In our previous studies, we investigated the GA contents in more than 1000 *G. uralensis* cultivars, and found there were only 14% samples, in which the GA content met the requirement of 2.0% regulated in *Pharmacopoeia of the Peoples Republic of China*. Therefore, improving the quality of *G. uralensis* cultivars has become a more and more important issue to satisfy the increasing demand of *G. uralensis*.

To date, the biosynthetic pathway of GA, the mevalonic acid (MVA) pathway, has been clarified (Fig. 1). Three key enzymes, 3-hydroxy-3-methylglutary CoA reductase (HMGR), squalene synthase (SQS), and *β*-amyrin synthase (*β*-AS), play very important role in this pathway. HMGR is the first rate-limiting enzyme involved in GA biosynthesis (Aquil *et al.,* 2009; Friesen & Rodwell, 2004; Harker *et al.,* 2003; Yang *et al.,* 1991). It catalyzes HMG-CoA and NADPH into MVA, which is an irreversible reaction. SQS situates in a branch point from farnesyl diphosphate (FPP) to triterpenoids and

plays an important role for regulating carbon flowing to triterpenoids (Lee *et al*., 2004; Lu *et al*., 2008; Seo *et al*., 2005). Up to date, at least two kinds of *SQS* genes have been found in *Arabidopsis thaliana* (Kribii *et al*., 1997; Mirjalili *et al*., 2011) and *Glycyrrhiza glabra* (Hayashi *et al*., 2003). *β*-AS is responsible for triterpene skeleton formation, it also situates in a branch point to catalyze 2, 3-oxidosqualene into *β*-amyrin (Hayashi *et al*., 2001; Wang *et al*., 2011). Many reports have demonstrated that there is a positive correlation between the accumulation of terpene and the dosage of *HMGR* (Chappell & Nable, 1987; Dai *et al*., 2011; Liu *et al*., 2014a), *SQS* (Liu *et al*., 2015; Lu *et al*., 2008), and *β-AS* genes (Liu *et al*., 2014b). Although the conclusions of the above researches were mainly obtained from the recombinant yeast and hairy roots systems, they still gave us an important inspiration. Over-expression of the functional genes involved in GA biosynthesis may improve the GA content in *G. uralensis* cultivars.

In the researches about over-expressing functional genes to control and regulate the metabolite production, the exogenous genes are often expressed in specific tissue by tissue specific promoter (Wang *et al*., 2015; Xue *et al*., 2016). Tissue specific expression can not only improve the accumulation of expression product, but also avoid or minimize unwanted effects (Jeon *et al*., 2015; Yu *et al*., 2015). In our previous study, we found that GA and *β-AS* gene were only synthesized or expressed in root and rhizome of *G. uralensis* (Liu Y & Liu CS, 2012).

We have already successfully cloned *G. uralensis HMGR* (*GuHMGR*) (Liu *et al*., 2013a), *G. uralensis SQS*1 (*GuSQS*1) (Liu *et al*., 2013b), and *G. uralensis BAS* (*GuBAS*) (Chen *et al*., 2013) in our previous work. Therefore in this paper, these three functional genes involved in GA biosynthetic pathway were selected as target genes and root-specifically over-expressed in *G. uralensis* callus cells, and regeneration of the transgenic *G. uralensis* plantlets was induced. The relationship between over-expression of the three functional genes and the content levels of GA and its epimer, isoglycyrrhizic acid (IGA) was analyzed in the regenerated transgenic *G. uralensis* plants. This work was intended to facilitate the molecular breeding research of *G. uralensis*, reveal the molecular mechanisms of GA biosynthesis, and improve the quality of *G. uralensis* cultivars.

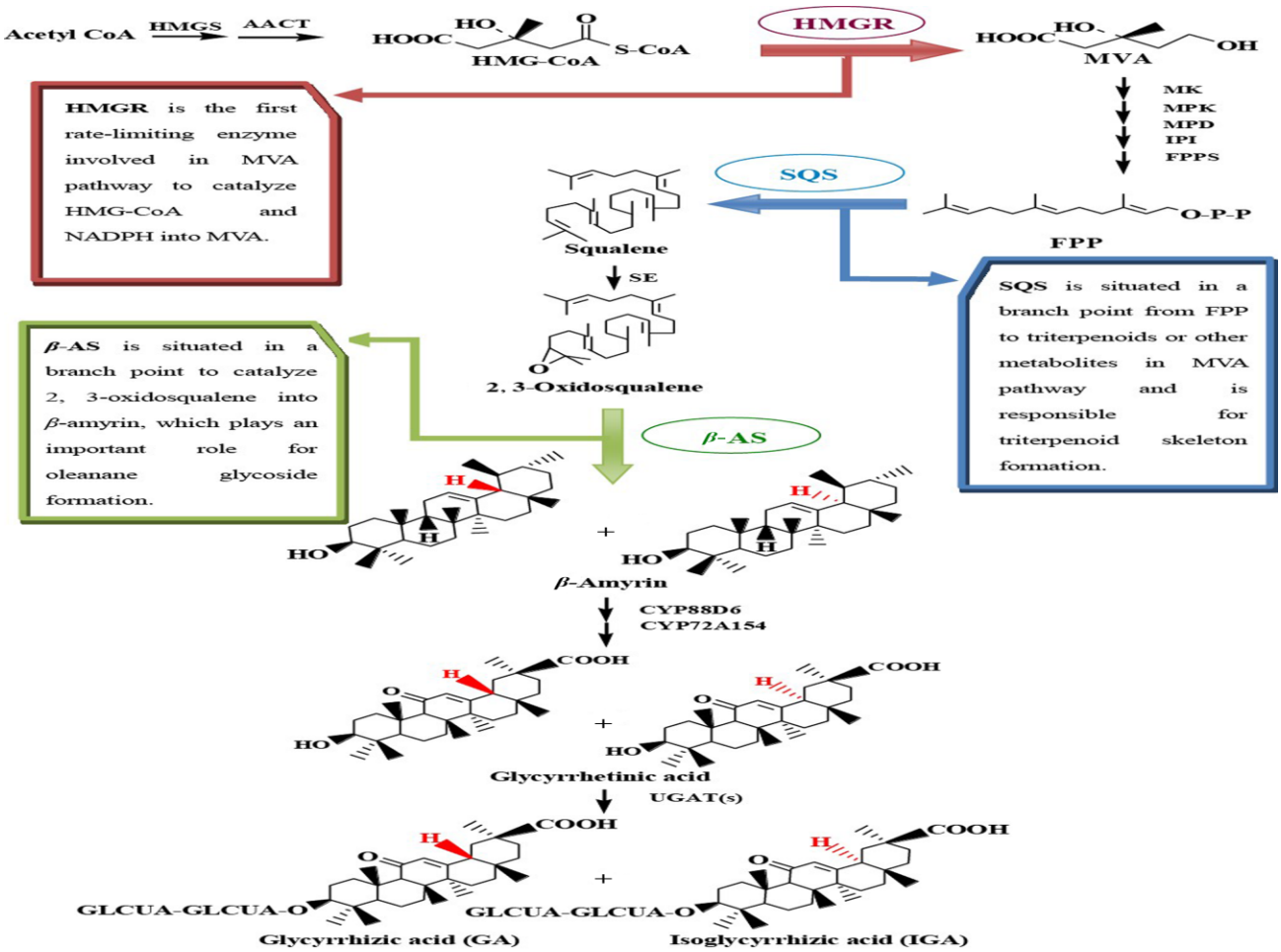

Fig. 1. The biosynthetic pathway of GA and IGA (MVA pathway) and the functions of HMGR (marked in red), SQS (marked in blue), and *β*-AS (marked in green).

Abbreviations: 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, MPD: mevalonate pyrophosphate decarboxylase, IPI: isopentenyl pyrophosphate isomerase, FPPS: farnesyl pyrophosphate synthase, FPP: farnesyl pyrophosphate, SQS: squalene synthase, SE: squaleneepoxidase.

Materials and Methods

Cloning of tobacco root-specific promoter TobRB7: Total DNA of tobacco was extracted from approximately 1g fresh plant leaves using plant DNA extraction kit (Beijing Biomed medical technology Co., LTD) and examined by spectrophotometry and electrophoresis in 1% (w/v) agarose gel. Based on Yamamoto's work (Yamamoto *et al.,* 1991), TobRB7 was amplified. The specific PCR primers for amplifying TobRB7 were as follows: forward primer (FP) was 5'-CCCCACCCGAAAGGAAATGATTC-3' and reverse primer (RP) was 5'-AATTGTTCTCACTAGAAAAATGCCCC-3'. The PCR cycling parameters were as follows: 94°C for 8min; 35 cycles of 94°C for 40 sec, annealing at 50°C for 40 sec, extension at 72°C for 60 sec; a final extension at 72°C for 8 min. The amplified fragment was purified and subcloned into pMD19-T (TaKaRa, Otsu, Shiga, Japan) and transferred into the disarmed *E. coli* DH5*α*, and then sequenced for correction.

Construction of the root specific expression vectors: The three exogenous genes, *GuHMGR* (Liu *et al.,* 2013a), *GuSQS*1 (Liu *et al.,* 2013b), and *GuBAS* (Chen *et al.,* 2013), were cloned in our previous work, and recombinant plasmids containing the three genes were kept in *E. coli* DH5*α* cells in our laboratory. The three recombinant plasmids were extracted by plasmid extraction kit (Beijing Biomed Medical Technology Co., Ltd, Beijing, China) and used as PCR template. And the recombinant pMD19-T vector with a TobRB7 was also extracted and used as PCR template.

Fig. 2a shows the construction strategy of root-specific expression vectors. The plant binary expression vector pCAMBIA1305.1 was used as vector skeleton. TobRB7 was used as promoter for the three exogenous genes, and NOS polyA, which located in pCAMBIA1305.1 vector, was used as terminator. Sequence analysis results did not support the double enzyme digestion method. So effusion kit (Baihui Biological Technology Co., Ltd, Beijing, China) was used to construct root-specific expression vectors. *Sph* I and *Spe* I were selected as insertion sites of fusion gene fragments, TobRB7-*GuHMGR*, TobRB7-*GuSQS*, and TobRB7-*GuBAS*. Four consecutive PCR reactions were necessary to obtain these fusion gene fragments. All of the primers used in the four PCR reactions were listed in Table 1. In the first PCR reaction, the recombinant pMD19-T vector with a TobRB7 was used as PCR template, primer No. 1 and No. 3 were used to obtain TobRB7, and the PCR cycling parameters were as follows: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 61°C for 45 sec, and 72°C for 120 sec; 72°C for 8 min. In the second PCR reaction, recombinant plasmids containing *GuHMGR*, *GuSQS*1, or *GuBAS* genes were used as template, primer No. 4 and No. 5 were used to obtain *GuHMGR*, *GuSQS*, and *GuBAS*, respectively, and the PCR cycling parameters were as follows: 94°C for 5 min; 35 cycles of 94°C for 45 sec, 50°C for 60 sec, and 72°C for 120 sec; 72°C for 8 min. In the third PCR reaction, TobRB7 obtained from the first PCR reaction and the three exogenous genes obtained from the second PCR reaction were used as template. Primer No. 3 and No. 4 were used as PCR primers and the PCR cycling parameters were as follows: 94°C for 5 min; 7 cycles of 94°C for 50 sec, 50°C for 60 sec, and 72°C for 100 sec; 72°C for 8 min. In the fourth PCR reaction, production of the third PCR reaction was used as template. Primer No. 2 and No. 6 were used to obtain the fusion gene fragments, and the PCR cycling parameters were as follows: 94°C for 5 min; 35 cycles of 94°C for 45 sec, 60°C for 60 sec, and 72°C for 120 sec; 72°C for 8 min. All of the amplified fragments were purified and ligated by effusion kit with linearized pCAMBIA1305.1, which was digested at *Sph* I and *Spe* I sites.

After construction, the three recombinant plasmids, pCA-TobRB7-HMGR, pCA-TobRB7-SQS, and pCA-TobRB7-BAS, were transferred into the disarmed *E. coli* DH5*α* (Beijing Biomed medical technology Co., LTD) by $CaCl_2$ freeze-thaw method, and the volume of recombinant gene product and *E. coli* solution was 5 μL and 50 μL, respectively. The transformed cells were cultured in none antibiotics Luria-Bertani (LB) liquid medium on 37ºC at 150 rpm for 45 min to recovery cells. And then *E. coli* cells were cultured on LB solid medium supplemented with 50 $mg \cdot L^{-1}$ kanamycin sulfate (Kan) on 37ºC for 12 ~ 16 h and the monoclonal colonies were validated strictly by PCR.

Table 1. Primers for construction of the root specific plant expression vectors.

Genes	No.	Sequences (5'-3')
Common use	No. 1	CGCCAGGGTTTTCCCAGTCACGA
	No. 2	GAGTCGACCTGCAGGCATGCCACCCGAAAGGAAATGATTC
	No. 5	GGATAACAATTTCACACAGG
GuHMGR	No. 3	GAACGTCCATTTTCCGACCCGTTCTCACTAGAAAAATGCCCC
	No. 4	GGGTCGGAAAATGGACGTTC
	No. 6	TTGATCGGGTACAGACTAGTGGAGGCTTTCGTTATTGGTC
*GuSQS*1	No. 3	ATCGCTCCCAAACTCCCCATGTTCTCACTAGAAAAATGCCCC
	No. 4	ATGGGGAGTTTGGGAGCGAT
	No. 6	TTGATCGGGTACAGACTAGTCGTGTTTGACCATTCGTTTC
GuBAS	No. 3	GCTATCTTCAGCCTCCACATGTTCTCACTAGAAAAATGCCCC
	No. 4	ATGTGGAGGCTGAAGATAGC
	No. 6	TTGATCGGGTACAGACTAGTTTAAGCTGGAGTGGAAGGCA

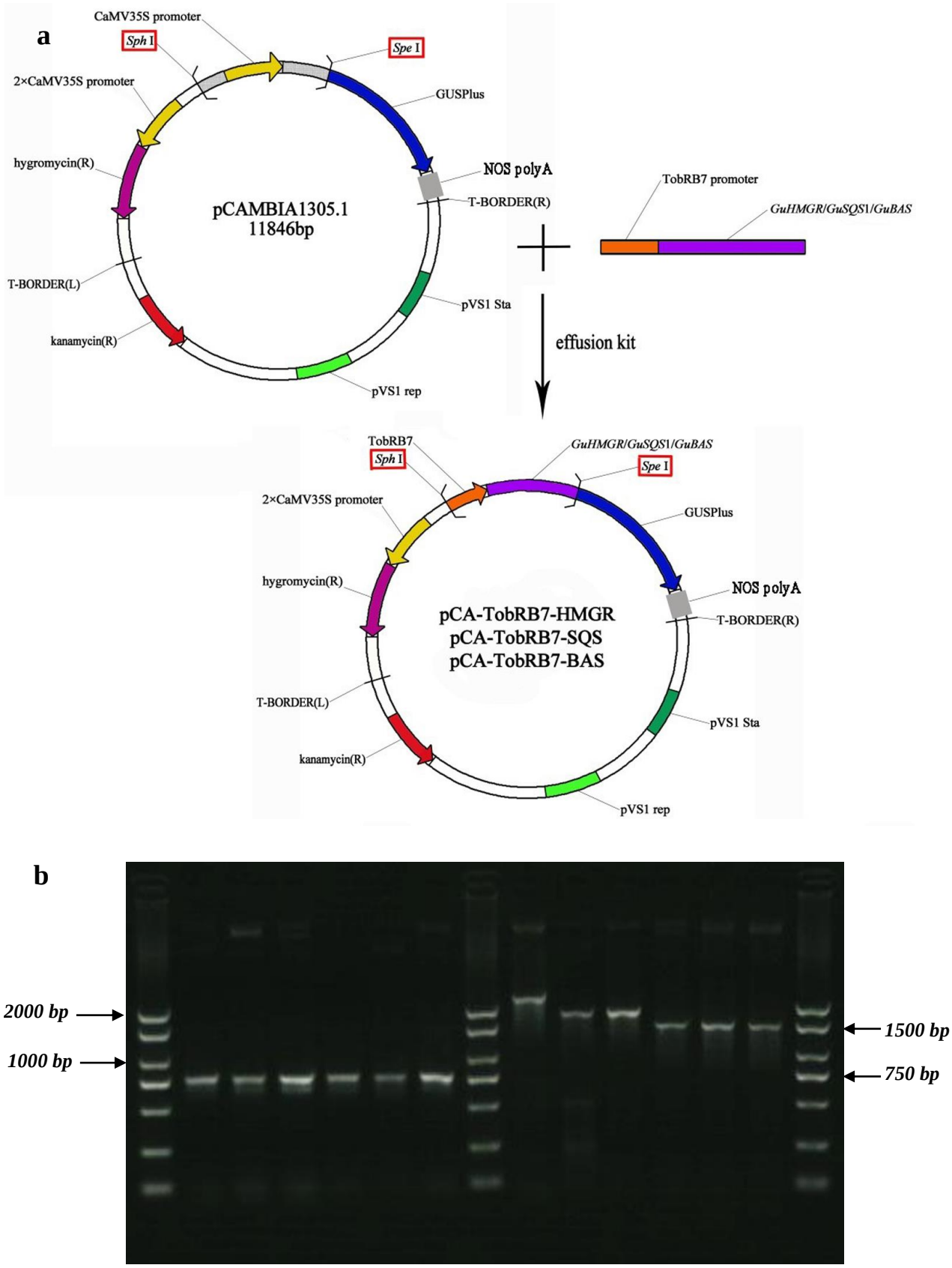

Fig. 2. The construction and verification of the three root-specific expression vectors, pCA-TobRB7-SQS, pCA-TobRB7-HMGR, and pCA-TobRB7-BAS. a shows the construction strategy, *Sph* I and *Spe* I outlined in red are the insertion sites of the fusion gene fragments, b shows PCR verification results of the three root specific expression vectors (No.1, No.8, and No.15 are DNA markers. No.2 ~ No.7 show the root-specific promoter TobRB7 in the three recombinant plasmids, No.9 shows *GuBAS* in the recombinant plasmids pCA-TobRB7-BAS, No.10 and No.11 show *GuHMGR* in pCA-TobRB7-HMGR, and No.12 ~ No.14 show *GuSQS*1 in pCA-TobRB7-SQS.)

Transformation and culture of recombinant *Agrobacterium tumefaciens* EHA105: After amplification in *E. coli* DH5*α*, recombinant plasmids, pCA-TobRB7-HMGR, pCA-TobRB7-SQS, and pCA-TobRB7-BAS, were extracted to transform competent *A. tumefaciens* EHA105. The recombinant plasmids (~50 ng) were mixed with 50 μL competent EHA105 cells solution. Standing 30 min on ice, the mixture was rapidly moved to liquid nitrogen freezing for 5 min, and then immediately transferred to water-bath on 37ºC for 3 min. Adding 1 mL Yeast Extract Broth (YEB) liquid medium (no antibiotics) to this mixture, the transformed EHA105 was cultured at 28ºC, 200 rpm for 3 ~ 5 h to recover cells. The EHA105 was then selected on YEB solid medium supplemented with 50 mg·L^{-1} Kan and 20 mg·L^{-1} hygromycin (Hyg) and validated strictly by PCR. Growth condition was set at 28ºC with a constant shaking speed of 180 rpm. When optical density (OD) of the suspension cultures of *A. tumefaciens* EHA105 was up to 0.7 at 600 nm, the *A. tumefaciens* EHA105 cells were collected by centrifugation and suspended in same cubage of liquid Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) and then used for plant transformation.

Transformation, induction, selection, and transplantation of regenerated transgenic *G. uralensis* plants: The *G. uralensis* seeds were surface-sterilized and germinated on MS solid medium in 16 h light/8 h dark. Then hypocotyls of 7-day-old seedlings were collected and floated in above-mentioned MS liquid medium containing *A. tumefaciens* EHA105 cells and incubated for 20 min at 28ºC. After infection, the explants were co-cultured on solid MS medium in dark at 25ºC for 48 hours, and then transferred onto solid MS medium with 1.0 mg·L^{-1} 2, 4-D, 0.5 mg·L^{-1} 6-BA, 50 mg·L^{-1} Kan, 20 mg·L^{-1} Hyg, and 500 mg·L^{-1} cefotaxim sodium (Cef) to eliminate *A. tumefaciens* EHA105 and induce resistant callus. The resistant callus was transferred onto solid MS medium with 0.5 mg·L^{-1} 6-BA, 0.1 mg·L^{-1} NAA, and 20 mg·L^{-1} Hyg and cultured at 25ºC with a 16 h light/8 h dark photoperiod for shoot organogenesis. The shoots were then cultured on solid 1/2MS medium with 0.6 mg·L^{-1} IAA for root organogenesis. The rooted plantlets growing in bottles were then transplanted to matrix material (50% vermiculite + 50% sandy soil) and irrigated by nutrient solution (1/10 MS inorganic and organic substance). After about 1~2 months, they were transplanted to the herb garden in Beijing University of Chinese Medicine. The regenerated transgenic *G. uralensis* plants were further examined by quantitative real-time PCR (qRT-PCR).

DNA isolation and copy number analysis: Genomic DNA from regenerated transgenic *G. uralensis* plants was isolated using plant DNA extraction kit (Beijing Biomed medical technology Co., LTD). qRT-PCR was used to determine the copy number of *GuHMGR*, *GuSQS*1 and *GuBAS* genes in regenerated transgenic *G. uralensis* plants. *Lectin* was selected as an internal standard gene for its intra-nonspecific, inter-specific and constant copy number characteristics (Liu *et al.*, 2014c). In this work, we obtained *Lectin* by PCR from total DNA of *G. uralensis* root. The primer pairs for amplifying *Lectin* were as follows: 5'-AAGAACGTCCTTCAAGTCCACAA-3', 5'-GCTATGAGGCTTCCACGAAGA-3'. The cycling parameters of PCR were: 94°C for 5 min; 30 cycles of 94°C for 50 s, 57°C for 50 s, 72°C for 50 s; a final extension at 72°C for 10 min. The construction of standard recombinant plasmids containing *GuHMGR*, *GuSQS*1, *GuBAS*, and *Lectin* genes, the drawing of standard curves, the calculation of copy numbers of *GuHMGR*, *GuSQS*1, and *GuBAS* genes were completely based on the description in our previous report (Liu *et al.*, 2014c). The specific primer pairs used in qRT-PCR are listed in Table 3. The cycling parameters of qRT-PCR were as follows: 95 °C for 10 min; 40 cycles of 95°C for 15 sec, 60°C for 60 sec, saving at 4°C.

RNA isolation and relative expression level analysis: RNA was extracted from roots of control and regenerated transgenic *G. uralensis* plants using RNAprep Plant Kit (TIANGEN Biotech (Beijing) Co., Ltd.), and DNA contamination was removed with DNaseI (TaKaRa) following the manufacturer's instructions. *Lectin* was used as reference gene (Liu *et al.*, 2014c). The specific primer pairs of qRT-PCR are given in Table 2. qRT-PCR cycling parameters were as follows: 95°C for 3 min; 40 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 50 sec. Each sample was parallelly measured for three times. Initial data were classed to two groups for T-test. Two independent sample T-test and Dunnett's T-test were applied in comparing the significance between regenerated transgenic *G. uralensis* samples and blank control, *α* value in this test was 0.05.

Assay of GA and IGA in the regenerated transgenic *G. uralensis* plants: The fresh root of each annual regenerated transgenic *G. uralensis* plant was collected and dried to a constant weight, respectively. Then 0.1 g powder of each sample was extracted with ultrasonic by 50 mL 50% methanol for 30 min in 50 mL volumetric flask. Samples were filtered by 0.45 μm filter membrane before high-performance liquid chromatography (HPLC) analysis. An individual stock solution of the standard compounds IGA (purity: 90.8%) and GA (purity: 98%) was prepared separately at a concentration of 0.010704 mg·mL^{-1} and 0.2164 mg·mL^{-1} in 50% methanol. The calibration curves were prepared at six concentration levels as 1 μL, 2 μL, 5 μL, 10 μL, 15 μL, and 20 μL.

An Agilent HPLC 1100 system (Agilent Technologies Inc, California, USA) consisted of a G1311A pump, a multisolvent delivery system, and a 6000LP ultraviolet (UV) detector was used to detect GA and IGA contents in each sample. The column was Agela Durashell C18-AM (250 mm×4.6 mm, 5μm, Bonna-Agela Technologies Inc, Tianjin, China). The column temperature was 50°C. The mobile phase consisted of 10 mol·L^{-1} ammonium perchlorate (A) and methyl alcohol (B) was applied to the gradient elution of 48% A/52% B. The UV detection wavelength was at 250 nm, the flow rate was 0.8 mL·min^{-1}, and the injection volume was 20 μL. The precision, repeatability, stability, recovery, were also investigated. Because HPLC data was non-normality hence nonparametric test was used to analyze the difference between different groups, *α* value in this test was 0.05, too.

Table 2. Primer pairs for relative expression level analysis.

cDNA		Sequences (5'-3')
GuHMGR	Forward primer (FP)	GATGCGGCAGTGACGGTAA
	Reverse primer (RP)	GCAATCCCTCCAAGGACTTC
*GuSQS*1	FP	TTTCGCACTCGTCATTCA
	RP	CTATCAGTATCGGCACCT
GuBAS	FP	CACCATGTTTTGTACTGCAC
	RP	AATCCACTTTCTTGCTCT
Lectin	FP	AAGAACGTCCTTCAAGTCCACAA
	RP	GCTATGAGGCTTCCACGAAGA

Table 3. Primer pairs for copy number determination.

Genes		Sequences (5'-3')
GuHMGR	FP	GATGCGGCAGTGACGGTAA
	RP	GCAATCCCTCCAAGGACTTC
*GuSQS*1	FP	ATGGCTGATAGGAGTTG
	RP	CATAATGCCAGTGTTCC
GuBAS	FP	CACCATGTTTTGTACTGCAC
	RP	AATCCACTTTCTTGCTCT
Lectin	FP	AAGAACGTCCTTCAAGTCCACAA
	RP	GCTATGAGGCTTCCACGAAGA

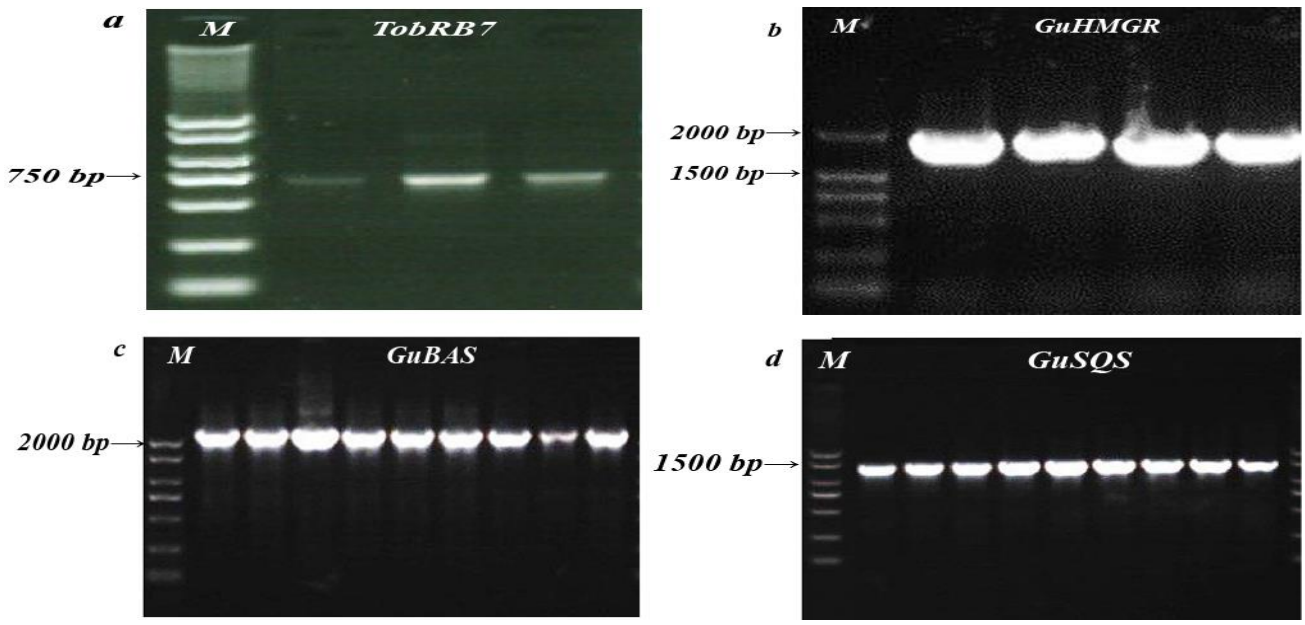

Fig. 3. PCR verification result of recombinant *A. tumefaciens* EHA105. a ~ d respectively shows TobRB7, *GuHMGR*, *GuBAS*, and *GuSQS*1 genes in recombinant *A. tumefaciens* EHA105.

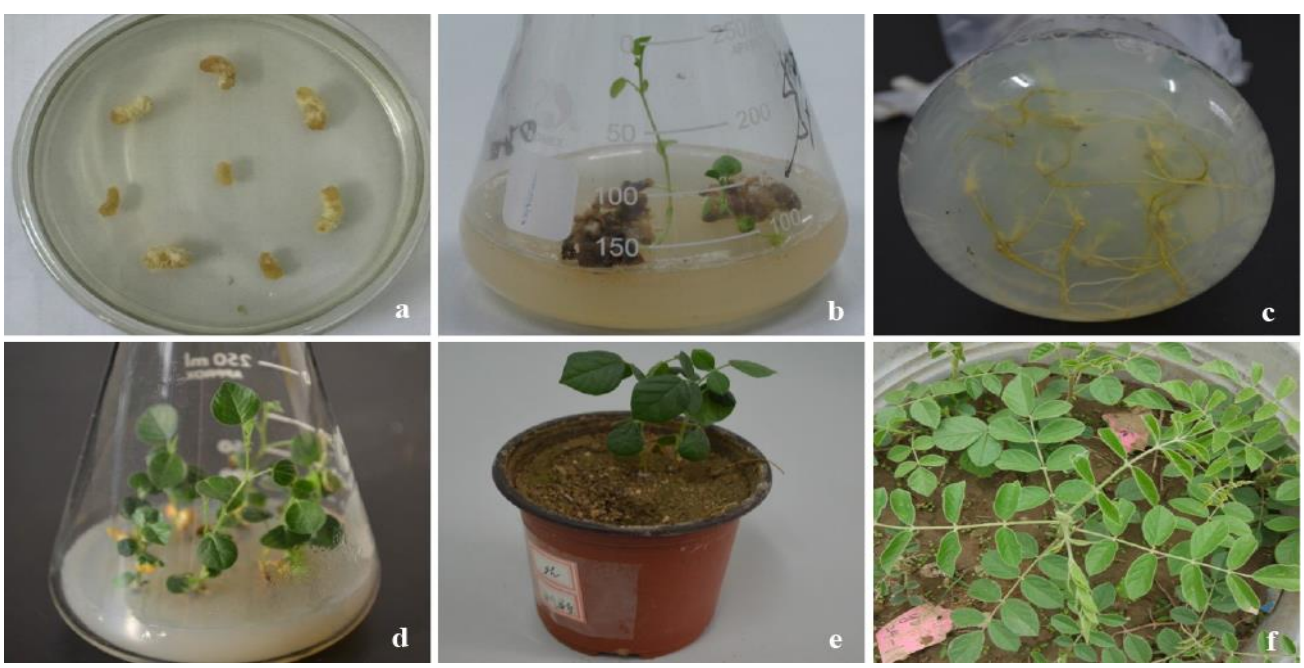

Fig. 4. Pictures of *G. uralensis* at different stages of transformation and regeneration. **a** shows the resistant callus after 2 weeks of culture, **b** shows shoot formation after 40 days of differentiation culture, **c** shows the strong roots after 50 days of rooting culture, **d** shows the healthy transgenic regenerated seedlings growing in the bottle, **e** shows the transgenic regenerated seedlings transplanted into 50% vermiculite and 50% sandy soil, and **f** shows transgenic *G. uralensis* plants transplanted to outdoor one year later.

Results

Cloning of tobacco root-specific promoter TobRB7: An approximate 730 bp fragment was obtained by PCR. The further sequencing result confirmed that the 721-bp fragment was tobacco root-specific promoter TobRB7. Blasting in GenBank (National Center for Biotechnology Information, USA), the result showed that sequence identity of this fragment and TobRB7 (Accession Number: JN656422) was 99%.

Construction of the root-specific expression vectors: According to the construction strategy described in Fig. 2a, three root-specific expression vectors were obtained. Fig. 2b shows the PCR verification results of the three root-specific expression vectors. Electrophoretic bands No.2 ~ No.7 show the 730 bp promoter TobRB7 in the three recombinant plasmids, band No.9 shows a 2289 bp *GuBAS* gene in the recombinant plasmid pCA-TobRB7-BAS, band No.10 and No.11 show a 1745 bp *GuHMGR* gene in pCA-TobRB7-HMGR, and band No.12 ~ No.14 show a 1239 bp *GuSQS*1 gene in pCA-TobRB7-SQS. Further sequencing results confirmed that the construction of the root-specific expression vectors was correct. By transformation experiments, the transgenic *A. tumefaciens* EHA105 was obtained and PCR and sequencing results validated strictly the correction of the positive clone. Fig. 3 shows PCR verification result of recombinant *A. tumefaciens* EHA105.

Induction, selection, culture, and transplantation of the regenerated transgenic *G. uralensis* plants: Fig. 4 shows the different stages of transformation and regeneration of *G. uralensis*. There were about 13% explants survived in infection and then dedifferentiated to resistant callus. Resistant callus (Fig. 4a) was obtained on solid MS medium in dark after two weeks of culture, and then transferred onto differential medium for shoots organogenesis. After about 40 days, regenerated shoots were induced and obtained (Fig. 4b). However, the shoots differentiation rate was very low, only about 7%. Strong shoots were then transferred onto rooting medium for root organogenesis. After about 50 days, strong and healthy roots were induced and got (Fig. 4c), and the root differentiation rate was 100%. After 2~4 weeks of culture, strong seedlings growing in bottles (Fig. 4d) were transplanted (Fig. 4e). One year later, healthy transgenic *G. uralensis* plants (Fig. 4f) were got. In the total transfer process, approximate 40% plantlets were dead. Surviving plantlets were well adapted to soil environment, there were no plant diseases, insect pests or degradation occurred. Ultimately, 3 control regenerated *G. uralensis* plants without infected by recombinant *A. tumefaciens* EHA105, 13 regenerated *G. uralensis* plants over-expressing *GuHMGR* gene root-specifically, 6 regenerated *G. uralensis* plants over-expressing *GuSQS*1 gene root-specifically, and 5 regenerated *G. uralensis* plants over-expressing *GuBAS* gene root-specifically were obtained.

Copy number of *GuHMGR*, *GuSQS*1, and *GuBAS* in the regenerated transgenic *G. uralensis* plants: PCR and sequencing results confirmed that the standard plasmids of qRT-PCR were correct. Melting curve analysis confirmed that the qRT-PCR primers of *GuHMGR*, *GuSQS*1, *GuBAS*, and *Lectin* were specific. qRT-PCR standard curves of *GuHMGR*, *GuSQS*1, *GuBAS*, and *Lectin* were $Y = -3.4581 X + 40.472$ (R^2 =0.999), $Y = -3.3914 X + 40.479$ (R^2 = 0.998), $Y = -3.1005 X + 38.171$ (R^2 = 0.996), and $Y = -3.0537 X + 36.164$ (R^2 = 0.999), respectively. Among the 13 regenerated *GuHMGR* transgenic *G. uralensis* plants, 3 samples had 2 copies, 6 samples had 3 copies, 2 samples had 4 copies, 1 sample had 5 or 6 copies of *GuHMGR* gene. Among the 6 regenerated *GuSQS*1 transgenic *G. uralensis* plants, 2 samples had 2 or 3 copies, 1 sample had 4 or 6 copies of *GuSQS*1 gene. Among the 5 regenerated *GuBAS* transgenic *G. uralensis* plants, 4 samples had 3 copies of *GuBAS* gene, and 1 sample had 5 copies of *GuBAS* gene.

Relative expression level of *GuHMGR*, *GuSQS*1, and *GuBAS* in the regenerated transgenic *G. uralensis* plants: The relative expression levels of *GuHMGR*, *GuSQS*1, and *GuBAS* in the regenerated transgenic *G. uralensis* plants are shown in Fig. 5. It was found that *GuHMGR*, *GuSQS*1, and *GuBAS* genes expressed normally in all of the transgenic *G. uralensis* plants. T-test results showed that the relative expression level of exogenous genes in transgenic plants was significantly different from which in the blank control ($T = 2.71$, $p<0.05$). According to Dunnett's T-test results, the relative expression level of *GuHMGR* gene in samples with 2 copies of *GuHMGR* (H1, H5, and H10) was not significant compared to blank control ($p>0.05$), and in samples with 3, 4, and 6 copies of *GuHMGR* (H2, H3, H4, H6, H7, H8, H11, H12, H13) was significantly higher ($p<0.05$) than that in blank control (Fig. 5a). Sample H9 with 5 copies of *GuHMGR* had the highest expression level. The relative expression level of *GuBAS* gene in sample with 5 copies of *GuBAS* (B2) was significantly higher ($p<0.05$) than that in blank control (Fig. 5b). And the relative expression level of *GuSQS*1 gene in samples with 2 copies of *GuSQS*1 (S3 and S6) was not significant ($p>0.05$) compared to that in blank control (Fig. 5c), while in samples with 3, 4, or 6 copies of *GuSQS*1 showed significant difference ($p<0.05$, Fig. 5c).

GA and IGA contents in the regenerated transgenic *G. uralensis* plants: Fig. 6a shows the HPLC chromatograms of reference substances (No. 1, marked in red), blank control (No. 2, marked in green), and one of the regenerated transgenic *G. uralensis* samples (No. 3, marked in purple). The retention time of IGA and GA is 14.100 min and 15.458 min, respectively. The contents of GA and IGA in each sample are showed in Fig. 6b ~ 6d. Blue indicates that the copy number of exogenous gene in the sample is two, green is three, purple is four, yellow is five, and red is six. It was found that the contents of GA and IGA in all of the regenerated transgenic *G. uralensis* samples were both higher than that in the blank control. And nonparametric test results (Table 4) showed that the difference between the contents of GA and IGA in the regenerated transgenic *G. uralensis* samples and in the blank control were statistically significant. It was also found that with the increase of copy number of *GuHMGR*, *GuSQS*1, and *GuBAS*, the contents of GA and IGA were both increased. Among the 13 samples over-expressing *GuHMGR* gene root-specifically,

the contents of GA (2.92%) and IGA (0.21%) were both the highest in sample H4 which had 6 copies of *GuHMGR* gene. Among the 6 samples over-expressing *GuSQS*1 gene root-specifically, the contents of GA (3.04%) and IGA (0.23%) were both the highest in sample S1 which had 6 copies of *GuSQS*1 gene. And among the 5 samples over-expressing *GuBAS* gene root-specifically, the contents of GA (2.39%) and IGA (0.16%) were both the highest in sample B2 which had 5 copies of *GuBAS* gene.

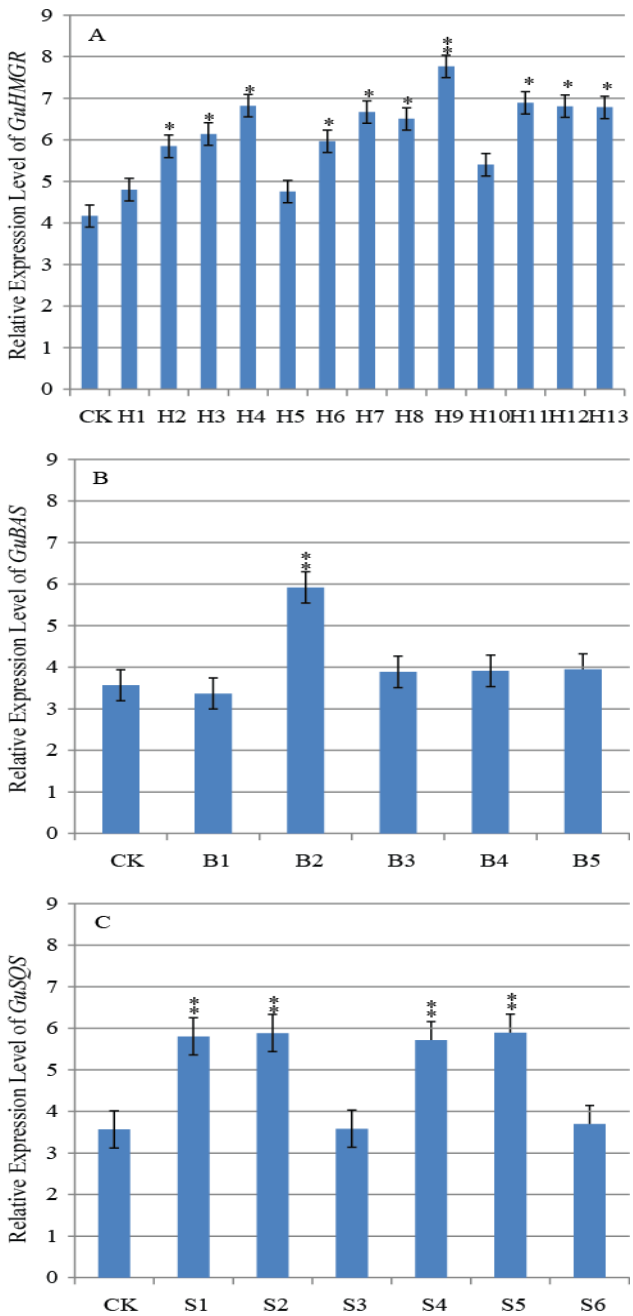

Fig. 5. Quantitative real-time RT-PCR transcription profile analysis of *GuHMGR*, *GuBAS*, *GuSQS*1. All RNA samples were isolated from fresh root of transgenic *G. uralensis* plants. Data are presented as the averages of three independent experiments conducted in triplicate. *Lectin* was used as reference gene. Error bars show standard deviations, * shows the T test results, ** represents $p<0.01$, and * represents $p<0.05$. a ~ c shows the relative expression levels of *GuHMGR*, *GuBAS*, and *GuSQS*1 in transgenic *G. uralensis* plants, respectively.

Discussion

In this paper, we obtained 13, 6, and 5 regenerated *G. uralensis* plants that root-specifically over-expressed *GuHMGR*, *GuSQS*1, and *GuBAS*, respectively. These regenerated transgenic *G. uralensis* plants contained different copy number of *GuHMGR*, *GuSQS*1, and *GuBAS* genes. We found that the contents of GA and IGA were both higher in the regenerated transgenic *G. uralensis* plants than that in blank control, and with the increase of the gene copy number, the contents were also increased. The highest GA and IGA contents in transgenic *G. uralensis* plants were about 3.1 times and 2.9 times higher than in the blank control, respectively. Biologically, the difference in gene copy number and expression level can lead to variations of enzyme content and finally affect the relevant products formation. Accordingly, with the increase of *GuHMGR*, *GuSQS*1, and *GuBAS* gene copy numbers, the content of corresponding enzymes also increase, resulting in the accumulation of GA and IGA. At the same time, the expression levels of the three genes in these transgenic *G. uralensis* plants were all higher than that in blank control, which also led to the higher accumulation of GA and IGA in the transgenic *G. uralensis*. The findings of this paper were intended to facilitate the molecular breeding of *G. uralensis* and improve the contents of GA and IGA in *G. uralensis* cultivars.

In other reports, Lu *et al*. placed the *GuSQS*1 gene under a CaMV 35S promoter and transferred it into *G. uralensis* by using *Agrobacterium rhizogenes* strain ACCC10060 (Lu *et al.*, 2008). Three transgenic hairy root lines were obtained and the HPLC analysis demonstrated that the highest GA content was about 2.6 times higher than the control hairy roots, which proved that *GuSQS*1 over-expression enhanced GA accumulation (Lu *et al.*, 2008). In our previous studies, we also wanted to reveal the effect of functional genes on the MVA pathway. We cloned *GuSQS*1 and *GuHMGR* genes, over-expressed them in *Pichia pastoris* GS115, and obtained 12 recombinant *P. pastoris* strains containing different copy number of *GuSQS*1 and *GuHMGR* genes. The HPLC analysis demonstrated that with the increase of copy number of *GuSQS*1 and *GuHMGR* genes, the content of ergosterol showed an increasing-decreasing-increasing pattern, which also proved that copy number variations (CNVs) of functional genes influenced the secondary metabolite production in MVA pathway (Liu *et al.*, 2014a; Liu *et al.*, 2015). However, the conclusions of the above researches were drawn in hairy root system or recombinant yeast system. Although these reports affirm the effect of functional genes on metabolite production in MVA pathway, it still needs further studies on plant level to reveal the impact of functional gene over-expression on GA biosynthesis.

GA and IGA are a pair of epimers, differed only in the C_{18}-H. In *Chinese Pharmacopoeia* GA is the stipulated marker component of *G. uralensis*. However, recent researches have demonstrated that IGA has a better lipotropy, a stronger anti-inflammatory activity, a higher

targeting and fewer adverse reactions comparing with GA (Chen *et al.*, 2014; Cheng *et al.*, 2009; Huang *et al.*, 2014; Zeng *et al.*, 2006), it gradually becomes a new evaluation index of high quality *G. uralensis*. Thus in this paper we also analyzed the content of IGA and found a positive correlation between the contents of IGA and GA. In our future studies we will further investigate the molecular mechanism of the formation of the important epimers, GA and IGA. And we intensely believe that *GuBAS* gene is the key entry point.

Conclusion

These transgenic *G. uralensis* plants had different copy number of *GuHMGR*, *GuSQS*1, and *GuBAS* genes, and their contents of GA and IGA were both higher than that in the blank control. Moreover, with the increase of the gene copy number, both the GA and IGA contents were increased. Therefore, root-specific over-expression of *GuHMGR*, *GuSQS*1, and *GuBAS* genes can enhance the accumulation of GA and IGA.

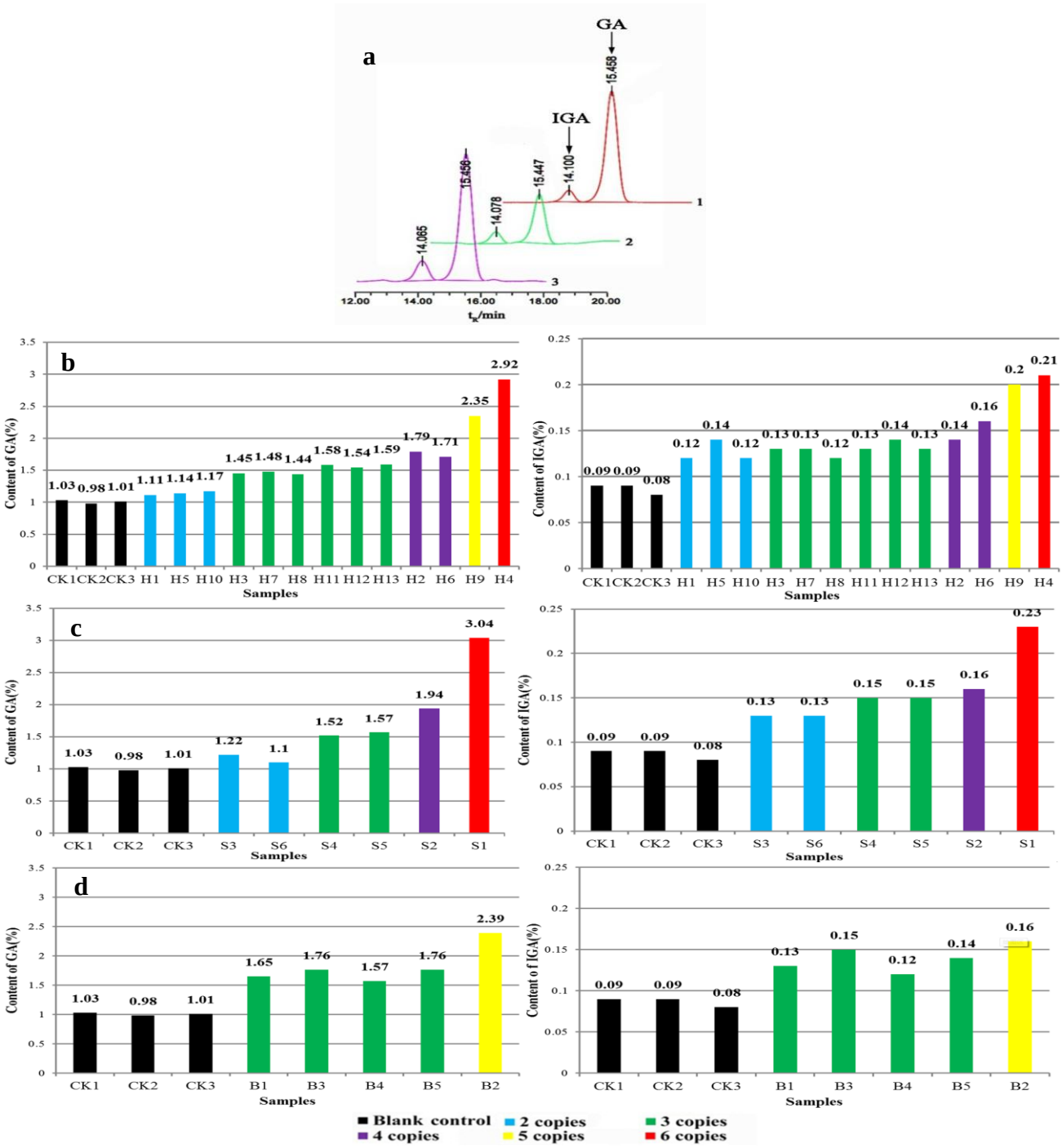

Fig. 6. HPLC analysis results. a shows the HPLC chromatograms of reference substances (No.1, marked in red), blank control (No.2, marked in green) and sample S1 root-specifically over-expressing *GuSQS*1 gene (No.3, marked in purple). The retention time of GA and IGA is 15.458 and 14.100, respectively. b ~ d show the contents of GA and IGA in blank control and samples root-specifically over-expressing *GuHMGR*, *GuSQS*1, and *GuBAS* genes, respectively.

Table 4. The nonparametric test results based on the contents of GA and IGA in the regenerated transgenic *G. uralensis* plants and blank control.

Compound	Items	Z value	*P* value
GA	Between all of the regenerated transgenic *G. uralensis* plants and blank control	-2.7397	0.0031
	Between samples root-specifically over-expressing *GuHMGR* and blank control	-2.5562	0.0053
	Between samples root-specifically over-expressing *GuSQS*1 and blank control	-2.1947	0.0297
	Between samples root-specifically over-expressing *GuBAS* and blank control	-2.1123	0.0173
IGA	Between all of the regenerated transgenic *G. uralensis* plants and blank control	-2.7751	0.0028
	Between samples root-specifically over-expressing *GuHMGR* and blank control	-2.5926	0.0048
	Between samples root-specifically over-expressing *GuSQS*1 and blank control	-2.2226	0.0131
	Between samples root-specifically over-expressing *GuBAS* and blank control	-2.0995	0.0179

Authors' contributions

BC Y and RY wrote this paper. YL, BC Y and RY constructed the three root-specific expression vectors and induced the regenerated transgenic *G. uralensis* plants. WD L analyzed the contents of GA and IGA in all of the samples. SZ and YS M helped to detect the copy number of *GuHMGR*, *GuSQS*1, and *GuBAS* genes. All of the authors read and approved the final version of the manuscript.

Conflict of interest

All authors declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (81503181).

References

Alqahtani, A., K. Hamid, A. Kam, K.H. Wong, Z. Abdelhak, V. Razmovski-Naumovski, K. Chan, K.M. Li, P.W. Groundwater and G.Q. Li. 2013. The pentacyclic triterpenoids in herbal medicines and their pharmacological activities in diabetes and diabetic complications. *Curr. Med. Chem*., 20: 908-931.

Anonymous. 2010. Pharmacopoeia of China, Part 1. Chemical Industry Press, Beijing.

Aquil, S., A.M. Husaini, M.Z. Abdin and G.M. Rather. 2009. Over expression of the HMG-CoA reductase gene leads to enhanced artemisinin biosynthesis in transgenic *Artemisia annua* plants. *Planta Med*., 75: 1453-1458.

Bhattacharjee, S., A. Bhattacharjee, S. Majumder, S.B. Majumdar and S. Majumdar. 2012. Glycyrrhizic acid suppresses Cox-2-mediated anti-inflammatory responses during *Leishmania donovani* infection. *J. Antimicrob Chemother*., 67: 1905-1914.

Chappell, J. and R. Nable. 1987. Induction of sesquiterpenoid biosynthesis in tobacco cell suspension cultures by fungal elicitor. *Plant Physiol*., 85: 469-473.

Chen, H.H., Y. Liu, X.Q. Zhang, X.J. Zhan and C.S. Liu. 2013. Cloning and characterization of the gene encoding *β*-amyrin synthase in the glycyrrhizic acid biosynthetic pathway in *Glycyrrhiza uralensis*. *Acta Pharmaceutica Sinica B*., 3: 416-424.

Chen, K.J., W.Y. Chen, X. Chen, Y.M. Jia, G.Q. Peng and L. Chen. 2014. Increased elimination of paclitaxel by magnesium isoglycyrrhizinate in epithelial ovarian cancer patients treated with paclitaxel plus cisplatin: a pilot clinical study. *Eur. J. Drug Metab. Pharmacokinet*, 39: 25-31.

Cheng,Y., J. Zhang, J. Shang and L. Zhang. 2009. Prevention of free fatty acid-induced hepatic lipotoxicity in HepG2 cells by magnesium isoglycyrrhizinate *In vitro*. *Pharmacology*, 84: 183-190.

Chueh, F.S., Y.T. Hsiao, S.J. Chang, P.P. Wu, J.S. Yang, J.J. Lin, J.G. Chung and T.Y. Lai. 2012. Glycyrrhizic acid induces apoptosis in WEHI-3 mouse leukemia cells through the caspase- and mitochondria-dependent pathways. *Oncol. Rep*., 28: 2069-2076.

Cynthia, W., Zabta, K., Shinwari, Imran, A., Riffat, N. 2011. Antibacterial activity in herbal products used in Pakistan. *Pakistan Journal of Botany*, 43: 155-162.

Dai, Z., G. Cui, S.F. Zhou, X. Zhang and L. Huang. 2011. Cloning and characterization of a novel 3-hydroxy-3-methylglutaryl coenzyme A reductase gene from *Salvia miltiorrhiza* involved in diterpenoid tanshinone accumulation. *J. Plant Physiol*., 168: 148-157.

Feng, L., M.M. Zhu, M.H. Zhang, R.S. Wang, X.B. Tan, J. Song, S.M. Ding, X.B. Jia and S.Y. Hu. 2013. Protection of glycyrrhizic acid against AGEs-induced endothelial dysfunction through inhibiting RAGE/NF-*κ*B pathway activation in human umbilical vein endothelial cells. *J. Ethnopharmacol*., 148: 27-36.

Friesen, J.A. and V.W. Rodwell. 2004. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. *Genome Biol*., 5: 248.

Harker, M., N. Holmberg, J.C. Clayton, C.L. Gibbard, A.D. Wallace, S. Rawlins, S.A. Hellyer, A. Lanot and R. Safford. 2003. Enhancement of seed phytosterol levels by expression of an N-terminal truncated Hevea brasiliensis (rubber tree) 3-hydroxy-3-methylglutaryl-CoA reductase. *Plant Biotechnol. J*., 1: 113-121.

Hayashi, H., P. Huang and K. Inoue. 2003. Up-regulation of soyasaponin biosynthesis by methyl jasmonate in cultured cells of *Glycyrrhiza glabra*. *Plant Cell Physiol*., 44: 404-411.

Hayashi, H., P. Huang, A. Kirakosyan, K. Inoue, N. Hiraoka, Y. Ikeshiro, T. Kushiro, M. Shibuya and Y. Ebizuka. 2001. Cloning and characterization of a cDNA encoding *β*-amyrin synthase involved in glycyrrhizin and

soyasaponin biosyntheses in licorice. *Biol. Pharm. Bull*., 24: 912-916.

Huang, X., J. Qin and S. Lu. 2014. Magnesium isoglycyrrhizinate protects hepatic L02 cells from ischemia/reperfusion induced injury. *Int. J. Clin. Exp. Pathol*., 7: 4755-4764.

Jeon, H.W., J.S. Cho, E.J. Park, K.H. Han, Y.I. Choi and J.H. Ko. 2015. Developing xylem-preferential expression of PdGA20ox1, a gibberellin 20-oxidase 1 from *Pinus densiflora*, improves woody biomass production in a hybrid poplar. *Plant Biotechnol. J*., 14: 1161-70.

Khan, R., A.Q. Khan, A. Lateef, M.U. Rehman, M. Tahir, F. Ali, O.O. Hamiza and S. Sultana. 2013. Glycyrrhizic acid suppresses the development of precancerous lesions via regulating the hyperproliferation, inflammation, angiogenesis and apoptosis in the colon of Wistar rats. *PLoS One*, 8: e56020.

Kratschmar, D.V., A. Vuorinen, C.T. Da, G. Wolber, D. Classen-Houben, O. Doblhoff, D. Schuster and A. Odermatt. 2011. Characterization of activity and binding mode of glycyrrhetinic acid derivatives inhibiting 11beta-hydroxysteroid dehydrogenase type 2. *J. Steroid. Biochem. Mol. Biol*., 125: 129-142.

Kribii, R., M. Arro, A.A. Del, V. Gonzalez, L. Balcells, D. Delourme, A. Ferrer, F. Karst and A. Boronat. 1997. 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. *Eur. J. Biochem*., 249: 61-69.

Lee, M.H., J.H. Jeong, J.W. Seo, C.G. Shin, Y.S. Kim, J.G. In, D.C. Yang and J.S. Yi. Choi YE, 2004. Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. *Plant Cell Physiol*., 45: 976-984.

Liu, Y., H.H. Chen, H. Wen, Y. Gao, L.Q. Wang and C.S. Liu. 2014b. Enhancing the accumulation of *β*-amyrin in *Saccharomyces cerevisiae* by co-expression of *Glycyrrhiza uralensis* squalene synthase 1 and *β*-amyrin synthase genes. *Yao Xue Xue Bao*., 49: 734-741.

Liu, Y. and C.S. Liu. 2012. Study on the spatial and temporal expression of *β*-AS gene of *Glycyrriza uralensis*. *Zhong Yao Cai*., 35: 528-531.

Liu, Y., Q.X. Xu, P.Y. Xi, H.H. Chen and C.S. Liu. 2013a. Cloning and characterization of a cDNA coding 3-hydroxy-3-methylglutary CoA reductase involved in glycyrrhizic acid. *Acta Pharmaceutica Sinica*., 48: 773-779.

Liu, Y., X. Zhan, W. Li, Y. Gao, H. Wen, H. Chen, L. Wang and C. Liu. 2014c. Copy number variations of functional genes influence contents of glycyrrhizic acid in *Glycyrrhiza uralensis*. *Acta Physiol Plant*, 36: 1433-1440.

Liu, Y., N. Zhang, H.H. Chen, Y. Gao, H. Wen, Y. Liu and C.S. Liu. 2013b. Cloning and characterization of two cDNA sequences coding squalene synthase involved in glycyrrhizic acid biosynthesis in *Glycyrrhiza uralensis*. *Lecture Notes in Electrical Engineering*, 269: 329-342.

Liu, Y., X. Zhu, W. Li, H. Wen, Y. Gao, Y. Liu and C. Liu. 2014a. Enhancing production of ergosterol in *Pichia pastoris* GS115 by over-expression of 3-hydroxy-3-methylglutaryl CoA reductase from *Glycyrrhiza uralensis*. *Acta. Pharm. Sin. B*., 4: 161-166.

Liu, Y., X.Q. Zhu, W.D. Li, H. Wen and C.S. Liu. 2015. Enhancing ergosterol production in *Pichia pastoris* GS115 by overexpressing squalene synthase gene from *Glycyrrhiza uralensis*. *Chin. J. Nat. Med*., 13: 338-345.

Lu, H., J. Liu, H. Zhang, T. Yin and S. Gao. 2008. Ri-mediated Transformation of *Glycyrrhiza uralensis* with a Squalene Synthase Gene (*GuSQS*1) for Production of Glycyrrhizin. *Plant Mol. Biol. Rep*., 26: 1-11.

Luo, L., Y. Jin, I.D. Kim and J.K. Lee. 2013. Glycyrrhizin attenuates kainic Acid-induced neuronal cell death in the mouse hippocampus. *Exp. Neurobiol*., 22: 107-115.

Ma, C., Z. Ma, X.L. Liao, J. Liu, Q. Fu and S. Ma. 2013. Immunoregulatory effects of glycyrrhizic acid exerts anti-asthmatic effects via modulation of Th1/Th2 cytokines and enhancement of CD4(+) CD25(+) $Foxp^{3+}$ regulatory T cells in ovalbumin-sensitized mice. *J. Ethnopharmacol*., 148: 755-762.

Matsumoto, Y., T. Matsuura, H. Aoyagi, M. Matsuda, S.S. Hmwe, T. Date, N. Watanabe, K. Watashi, R. Suzuki, S. Ichinose, K. Wake, T. Suzuki, T. Miyamura, T. Wakita and H. Aizaki. 2013. Antiviral activity of glycyrrhizin against hepatitis C virus in vitro. *PLoS One*, 8: e68992.

Mirjalili, M.H., E. Moyano, M. Bonfill, R.M. Cusido and. J. Palazón. 2011. Overexpression of the *Arabidopsis thaliana* squalene synthase gene in *Withania coagulans* hairy root cultures. *Biol. Plantarum*., 55: 357-360.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*., 15: 473-497.

Raikkonen, K., J.R. Seckl, K. Heinonen, R. Pyhala, K. Feldt, A. Jones, A.K. Pesonen, D.I. Phillips, J. Lahti, A.L. Jarvenpaa, J.G. Eriksson, K.A. Matthews, T.E. Strandberg and E. Kajantie. 2010. Maternal prenatal licorice consumption alters hypothalamic-pituitary-adrenocortical axis function in children. *Psychoneuroendocrinology*, 35: 1587-1593.

Rasool, M., J. Iqbal, A. Malik, H.S. Ramzan, M.S. Qureshi, M. Asif, M.H. Qazi, M.A. Kamal, A.G. Chaudhary, M.H. Al-Qahtani, S.H. Gan and S. Karim. 2014. Hepatoprotective effects of *Silybum marianum* (Silymarin) and *Glycyrrhiza glabra* (Glycyrrhizin) in combination: A Possible Synergy. *Evid. Based Complement Alternat Med*., 2014: 641597.

Seo, J.W., J.H. Jeong, C.G. Shin, S.C. Lo, S.S. Han, K.W. Yu, E. Harada, J.Y. Han and Y.E. Choi. 2005. Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation. *Phytochemistry*, 66: 869-877.

Smirnov, V.S., V.V. Zarubaev, P.M. Anfimov and A.A. Shtro. 2012. Effect of a combination of glutamyl-tryptophan and glycyrrhizic acid on the course of acute infection caused by influenza (H3H2) virus in mice. *Vopr. Virusol*., 57: 23-27.

Tripathi, M., B.K. Singh and P. Kakkar. 2009. Glycyrrhizic acid modulates t-BHP induced apoptosis in primary rat hepatocytes. *Food Chem. Toxicol*., 47: 339-347.

Wang, L., Q. Lu, X. Wen and C. Lu. 2015. Enhanced sucrose loading improves rice yield by increasing grain size. *Plant Physiol*., 169: 2848-2862.

Wang, Z., O. Guhling, R. Yao, F. Li, T.H. Yeats, J.K. Rose and R. Jetter. 2011. Two oxidosqualene cyclases responsible for biosynthesis of tomato fruit cuticular triterpenoids. *Plant Physiol*., 155: 540-552.

Wei, S.L., W.Q. Wang, J.Y. Wang, Z.R. Sun, C.S. Liu, H. Wang, Z.G. Yang and B.C. Wu, 2012. Preliminary study in glycyrrhizin content and its influencing factors of wild and cultivated in different region of China. *Zhongguo Zhong Yao Za Zhi*., 37: 1341-1345.

Xue, G.P., A.L. Rae, R.G. White, J. Drenth, T. Richardson and C.L. McIntyre. 2016. A strong root-specific expression system for stable transgene expression in bread wheat. *Plant Cell Rep*., 35: 469-481.

Yamamoto, Y.T., C.G. Taylor, G.N. Acedo, C.L. Cheng and M.A. Conkling. 1991. Characterization of cís-acting sequences regulating root-specific gene expression in tobacco. *The Plant Cell*, 3: 371-382.

Yang, Z., H. Park, G.H. Lacy and C.L. Cramer. 1991. Differential activation of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes by wounding and pathogen challenge. *Plant Cell*, 3: 397-405.

Yu, Z.H., Y.N. Han and X.G. Xiao. 2015. A PPO promoter from betalain-producing red swiss chard, directs petiole- and root-preferential expression of foreign gene in anthocyanins-producing plants. *Int. J. Mol. Sci*., 16: 27032-27043.

Zeng, C.X., Q. Yang and Q. Hu. 2006. A comparison of the distribution of two glycyrrhizic acid epimers in rat tissues. *Eur. J. Drug Metab. Pharmacokinet*, 31: 253-258.

(Received for publication 18 December 2017)