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Original Article

Over-expressing root-specific β-amyrin synthase gene increases glycyrrhizic acid content in hairy roots of *glycyrrhiza uralensis*

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ABSTRACT

Objsective: *Glycyrrhizia uralensis*, one of the most widely-used traditional Chinese medicines, is mainly cropped in China. However, many cultivars are less in glycyrrhizic acid than Chinese Pharmacopoeia requires. In this paper, we improved glycyrrhizic acid by regulating β-amyrin synthase gene (*GuBAS)*.
Methods: Tobacco root-specific promoter *TobRB*7 and *GuBAS* cDNA were obtained and combined with linearized pCAMBIA1305.1 to construct root-specific plant expression vector which was later transformed into *Agrobacterium rhizogenes* ACCC10060 by electrotransformation. The cotyledons and hypocotyls of *G. uralensis* were infected by the recombinant *A. rhizogenes* ACCC10060 to induce hairy roots. The GA content was quantified by HPLC.
Results: The PCR and sequencing results both showed that three transgenic hairy root lines were obtained. The copy number of *GuBAS* in these transgenic hairy roots was intended by qRT-PCR to be 3, 7, and 4. GA was detected by HPLC, and the results showed that GA was present in the three transgenic hairy roots, while absent in wild hairy roots.
Conclusion: Over-expressing *GuBAS* root-specifically in hairy roots of *G. uralensis* enhanced GA accumulation.

1. Introduction

The roots and rhizomes of *Glycyrrhiza uralensis* Fisch., one of the most important herbal medicines in China, have been used in traditional Chinese medicine (TCM) for over 2000 years. It has functions of relieving coughing, nourishing *qi*, tonifying spleen and stomach, alleviating pain, and eliminating phlegm (Chinese Pharmacopoeia Committee, 2015). Glycyrrhizic acid (GA), the most important compound in *G. uralensis*, is responsible for various pharmacological activities such as antitumor (Chueh et al., 2012), anti-inflammatory (Ni et al., 2011), antiviral (Duan & Ji, 2007; Huang et al., 2012), immunoregulatory (Ma et al., 2013), antidiabetic (Sen, Roy, & Chakraborti, 2011), hepatoprotective activities (Rasool et al., 2014), etc. (Kratschmar et al., 2011; Räikkönen et al., 2010). GA is also frequently used as industrial raw materials and tobacco additives. Therefore, the demand for GA has yearly increased in industry.

As a result of the progressive decline of wild *G. uralensis* resources in China, Chinese government strictly restricts the collection and commercial uses of wild *G. uralensis* and cultivars have become the main source of *G. uralensis*. However, based on an investigation of the GA content in over 1000 samples of *G. uralensis* cultivars, we found that GA content was less than the level that *Chinese pharmacopoeia* regulates. Therefore, it is important to improve GA content or breeding desired cultivars using biological technology.

In *G. uralensis*, many important enzymes are involved in the biosynthesis of GA, of which β-amyrin synthase (β-AS), an oxidosqualene cyclase (OSC), catalyzes 2,3-oxidosqualene into β-amyrin, responsible for triterpene skeleton formation (Fig. 1) (Hayashi et al., 2001; Wang et al., 2011). Many reports have demonstrated that over-expression of functional genes involved in triterpene metabolic pathway, e.g., *HMGR* (Chappell & Nable, 1987; Dai, Cui, Zhou, Zhang, & Huang, 2011; Liu et al., 2014) and *SQS* (Liu, Zhu, Li, Wen, & Liu, 2015; Lu, Liu, Zhang, Yin, & Gao, 2008) improved the content of triterpenes. These reports lead to a hypothesis that over-expression of β-*AS* gene may enhance the accumulation of GA.

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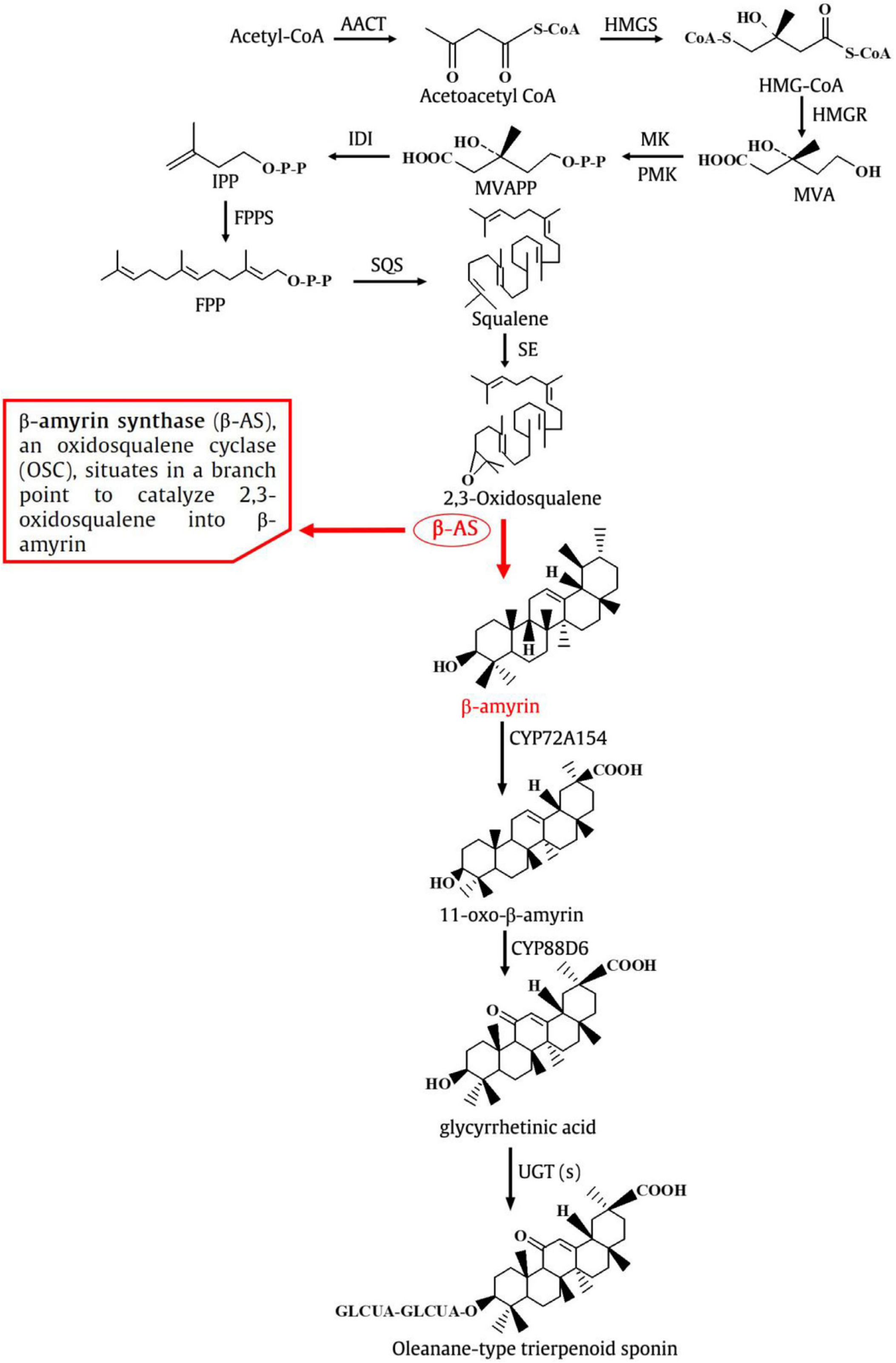

β-amyrin synthase (β-AS), an oxidosqualene cyclase (OSC), situates in a branch point to catalyze 2,3-oxidosqualene into β-amyrin

Fig. 1. Biosynthetic pathway of triterpenes.

Tissue specific expression is a biological technology that can not only improve the accumulation of expression product, but also avoid or minimize side effects (Jeon et al., 2016; Yu, Han, & Xiao, 2015). Therefore, when exogenous genes are over-expressed to control and regulate the metabolite production, they are often expressed in specific tissue by tissue specific promoter (Wang, Lu, Wen, & Lu, 2015; Xue et al., 2016). Our previous study showed that β-*AS* gene only expressed in root and rhizome of *G. uralensis* (Liu & Liu, 2012). Therefore, in this paper, *G. uralensis* β-*AS* (*GuBAS*) was root-specifically over-expressed in *G. uralensis* hairy roots, and the

GA content was assayed. The results of this paper are intended to facilitate β-*AS* gene function research and expand the GA source.

2. Materials and methods

2.1. Construction of root specific expression vectors

2.1.1. Cloning of tobacco root-specific promoter TobRB7

Total tobacco DNA was extracted from 1 g fresh leaves using plant DNA extraction kit (Beijing Biomed Medical Technology Co., Ltd.). The PCR forward primer (FP) and reverse primer (RP) were 5′-CCCCACCCGAAAGGAAATGATTC-3′ and 5′-AATTGTTCTCACTAGAAAAATGCCCC-3′, respectively. Thirty-five cycles of amplification were carried out at 94 °C for 40 s, 50 °C for 40 s, 72 °C for 60 s with an 8 min final extension at 72 °C. The purified PCR-amplified product was subcloned into pMD19-T (Takara, Otsu, Shiga, Japan) and sequenced.

2.1.2. Isolation and cloning of GuBAS

Total RNA was extracted from fresh *G. uralensis* roots using RNA prep Plant Kit (TIANGEN Biotech (Beijing) Co., Ltd.), and used as template to synthesize cDNA. A pair of specific primers was designed based on Hayashi's research (Hayashi et al., 2001) as follows: FP was 5′-ATGTGGAGGCTGAAGATAGCG-3′, and RP was 5′-TTAAGCTGGAGTGGAAGGCAA-3′. The PCR cycling parameters were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 60 °C for 60 s, and 72 °C for 3 min; 72 °C for 10 min. The purified PCR-amplified product was subcloned into pMD19-T (Takara, Otsu, Shiga, Japan) and sequenced.

2.1.3. Construction of plant root-specific expression vector

Fig. 2 showed the construction strategy of root-specific expression vector using effusion kit (Dongguan Baihui biological technology Co., Ltd.), pCAMBIA1305.1 was the vector skeleton, and *Sph* I and *Spe* I are the insertion sites. Four consecutive PCR reactions were necessary to obtain the fusion gene fragment, *TobRB7-GuBAS*. All 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

Table 1
Primers for construction of root-specific plant expression vector.

No.	Sequences (5′–3′)
1	CGCCAGGGTTTTCCCAGTCACGA
2	GAGTCGACCTGCAGGCATGCCACCCGAAAGGAAATGATTC
3	GCTATCTTCAGCCTCCACATGTTCTCACTAGAAAAATGCCCC
4	ATGTGGAGGCTGAAGATAGC
5	GGATAACAATTTCACACAGG
6	TTGATCGGGTACAGACTAGTTTAAGCTGGAGTGGAAGGCA

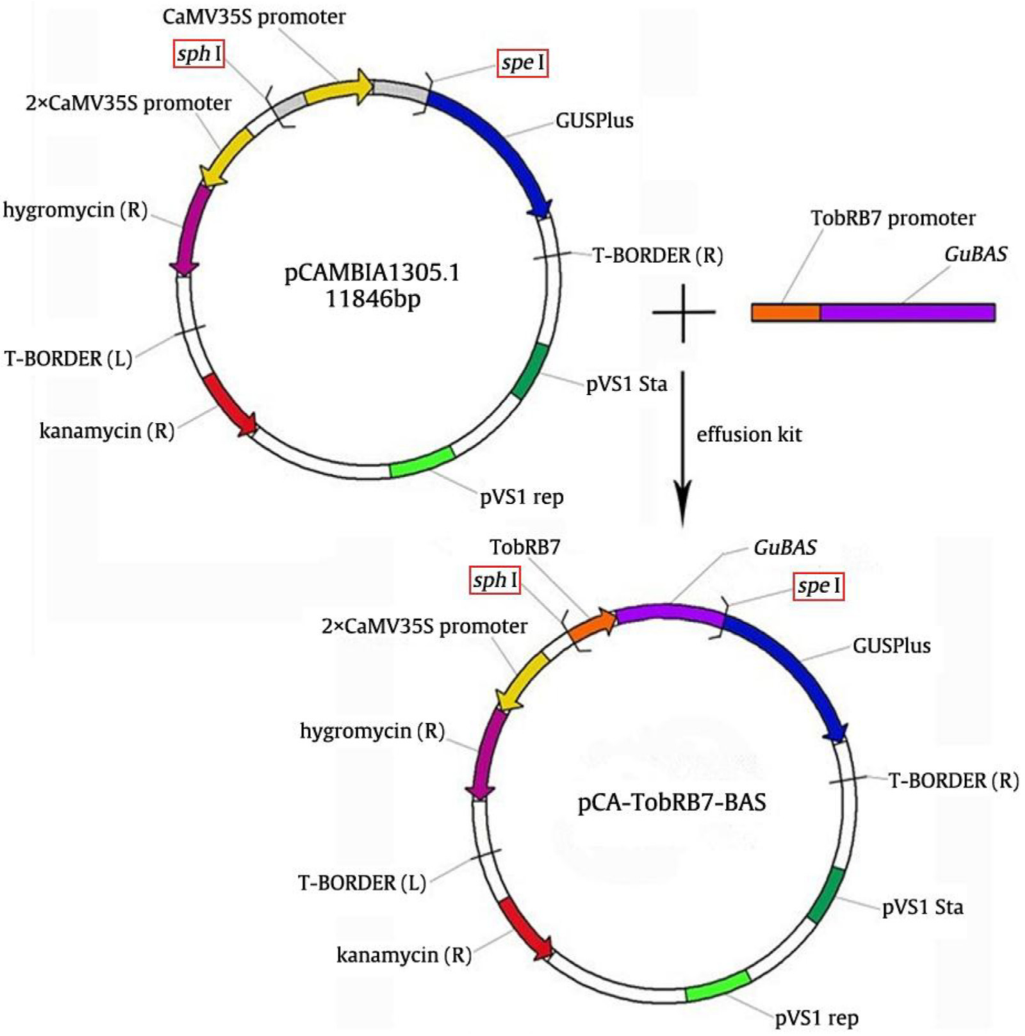

Fig. 2. Construction strategy of root-specific expression vector pCA-TobRB7-BAS.

No. 1 and No. 3 were used to obtain *TobRB*7, and the PCR cycling parameters were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 61 °C for 45 s, and 72 °C for 120 s; 72 °C for 8 min. In the second PCR reaction, recombinant plasmid containing *GuBAS* gene was used as template, primer No. 4 and No. 5 were used to obtain *GuBAS* gene, and the PCR cycling parameters were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 120 s; 72 °C for 8 min. In the third PCR reaction, *TobRB*7 obtained from the first PCR reaction and *GuBAS* gene 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 s, 50 °C for 60 s, and 72 °C for 100 s; 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 s, 60 °C for 60 s, and 72 °C for 120 s; 72 °C for 8 min. All the amplified fragments were purified and ligated by effusion kit with linearized pCAMBIA1305.1, which was digested at *Sph* I and *Spe* I sites. The recombinant plasmid, pCA-TobRB7-BAS, was transferred into the disarmed *Escherichia coli* DH5α, and then sequenced for correct insertion.

2.2. Transformation and culture of recombinant Agrobacterium rhizogenes ACCC10060

After amplification in *E. coli* DH5α, pCA-TobRB7-BAS was used to transform *A. rhizogenes* ACCC10060 by electrotransformation, which was then selected on YEB medium supplemented with 50 mg/L kanamycin sulfate (Kan) and 20 mg/L hygromycin (Hyg). The wild *A. rhizogenes* ACCC10060 was cultured in YEB medium without Kan and Hyg. Growth condition was set at 28 °C with a constant shaking speed of 180 rpm. Plasmids of the transgenic and wide strains were extracted by Plasmid extraction kit (Beijing Biomed medical technology Co., Ltd.) and then validated strictly by PCR. The PCR primers are listed in Table 2. The PCR cycling parameters of *TobRB*7 and *GuBAS* were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 50 s, 52 °C for 50 s and 72 °C for 90 s; 72 °C for 10 min. The PCR cycling parameters of *A. rhizogenes rol*C were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; 72 °C for 10 min. When optical density (OD) of the suspension cultures of *A. rhizogenes* ACCC10060 was up to 0.7 at 600 nm, the *A. rhizogenes* ACCC10060 cells were collected by centrifugation and suspended in same cubage of liquid Mruashige and Skoog (MS) medium and then used for plant transformation.

2.3. Transformation, induction, selection and confirmation of G. uralensis hairy roots

The *G. uralensis* seeds were surface-sterilized and germinated on MS medium containing 2.5% sucrose and 0.7% agar for 7 d (16-h light/8-h dark). Cotyledons and hypocotyls of 7-day-old seedlings were cut and incubated in above-mentioned *A. rhizogenes* ACCC10060 suspension for 20 min at 28 °C, and then transferred onto MS medium in dark at 25 °C for 2 d. Then the explants were washed with sterile water and transferred onto fresh solid MS medium with 400 mg/L cefotaxime sodium (Cef) to eliminate *A. rhizogenes* ACCC10060 and subcultured every 7 d in the same medium until adventitious roots were 5 cm in length. These hairy roots were excised from explants and transferred to MS solid medium with 200 mg/L Cef and subcultured every 10 d until the residual bacteria were completely killed. The hairy roots were further examined by PCR for confirmation of the genetic transformation. Then liquid cultures were established with 5-cm-long tips from 40-day-old hairy roots in liquid MS medium at 25 °C on a rotary shaker with a constant shaking speed of 110 rpm in the dark.

Table 2
Primers for PCR validation.

Genes	Names	Sequences (5′–3′)
*TobRb*7	TF	CACCCGAAAGGAAATGATTC
	TR	GTTCTCACTAGAAAAATGCCCC
BAS	BF	ATGTGGAGGCTGAAGATAGC
	BR	TTAAGCTGGAGTGGAAGGCA
*rol*C	RF	CATATATGCCAAATTTACACTAG
	RR	GTTAACAAACTAGGAAACAGG

2.4. Analysis of copy number of β-AS gene in transgenic hairy roots by real-time PCR

Lectin gene from *G. uralensis* was selected as internal control gene for real-time PCR. Primers for amplifying *Lectin* gene were as follows: LF: 5′-AAGAACGTCCTTCAAGTCCACAA-3′, LR: 5′-GCTATGAGGCTTCCACGAAGA-3′. Primers for amplifying *GuBAS* gene were as follows: BF: 5′- CACCATGTTTTGTACTGCAC-3′, BR: 5′-AATCCACTTTCTTGCTCT-3′. The PCR cycling parameters were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 50 s, 57 °C for 50 s, 72 °C for 50 s; And a final extension at 72 °C for 10 min. The purified PCR-amplified fragments were subcloned into pMD19-T and then transformed into disarmed *E. coli* DH5α. The standard plasmid pMD19-T-BAS and pMD19-T-Lectin were obtained and diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 copy number/μL. The primers, BF, BR, LF, and LR, were used in real-time PCR, and the parameters were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and storing at 4 °C. Taking Ct value as *Y*-axis and log value of concentration of standard plasmid as *X*-axis, the standard curve was established. All the transgenic hairy roots were amplified respectively in the same conditions of real-time PCR, and each sample was repeated three times to ensure the accuracy. The data was substituted into the standard curve to calculate the copy number of *GuBAS* gene and *Lectin* gene. The ratio of *Y* value of *GuBAS* gene to *Y* value of *Lectin* gene was the copy number of *GuBAS* gene in each hairy root sample.

2.5. Assay of GA content in G. uralensis hairy roots

After culturing for 40 d, the *G. uralensis* hairy roots were collected and dried to a constant weight at 60 °C. Then 0.1 g powder of each hairy root sample was extracted with ultrasonic by 50 mL 50% methanol for 30 min in a 50 mL volumetric flask, and filtered by 0.45 μm filter membrane before high-performance liquid chromatography (HPLC) analysis. An individual stock solution of the standard compound GA (purity: 98%) was prepared at a concentration of 0.2164 mg/mL in 50% methanol. The standard curve was established at six sample sizes of 1 μL, 2 μL, 5 μL, 10 μL, 15 μL, and 20 μL. An Agilent HPLC 1100 system with a G1311A pump and a 6000LP ultraviolet (UV) detector (Agilent Technologies Inc, California, USA) and an Agela Durashell C18-AM column (250 mm × 4.6 mm, 5 μm, Bonna-Agela Technologies Inc, Tianjin, China) were used to detect the GA content in each sample. The mobile phase consisted of 10 mol/L ammonium perchlorate (A), and methyl alcohol (B) were applied to the gradient elution of 48% A/52% B. The column temperature was 50 °C, the UV detection wavelength was 250 nm, the flow rate was 0.8 mL/min, and the injection volume was 20 μL. The precision, repeatability, stability, and recovery were also investigated.

3. Results

3.1. Construction of root specific expression vector

Two sequences with a length of 721 bp and 2289 bp were obtained by PCR, respectively. Through online Basic Local Alignment Search Tool (BLAST) analysis, the 721 bp sequence shared a 99% similarity with *TobRB7* (GenBank accession No. S45406.1), and the 2289 bp sequence shared a 99% similarity with *GuBAS* cDNA sequence (GenBank accession No. AB037203.1), which indicated that the two sequences obtained by PCR were *TobRB7* and *GuBAS*, respectively. According to the construction strategy described in Fig. 2, the root specific expression vector, pCA-TobRB7-BAS, was established. Fig. 3 shows the PCR verification results of *TobRB7* (No.1–3) and *GuBAS* (No.4–9) in pCA-TobRB7-BAS.

3.2. Construction of recombinant Agrobacterium rhizogenes ACCC10060

The PCR validation results of the single colonies showed that they were all positive (Fig. 4). The further sequencing results showed that the length of the three sequences was 580 bp, 721 bp, and 2289 bp, respectively. Through online BLAST analysis, the 580 bp sequence shared a 99% similarity with *A. rhizogenes rolC* gene (GenBank accession No. DQ160187.1), the 721 bp sequence fragment shared a 99% similarity with *TobRB7* (GenBank accession No. S5406.1), and the 2289 bp sequence shared a 99% similarity with *GuBAS* cDNA sequence (GenBank accession No. FJ627179.1), which demonstrated that the construction of recombinant *A. rhizogenes* containing *GuBAS* gene was successful.

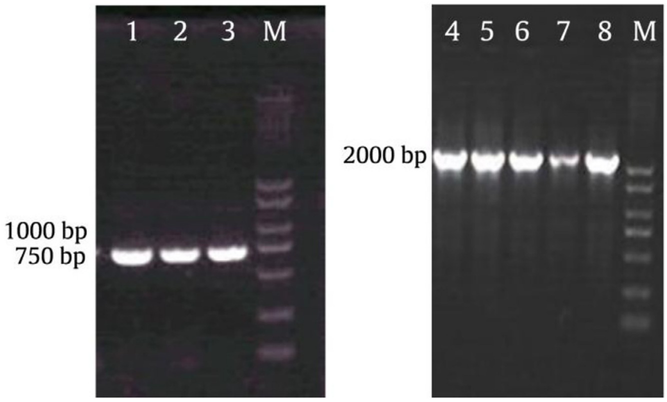

Fig. 3. *PCR verification results of root specific expression vector.* M: DNA marker, Lanes 1-3: root-specific promoter *TobRB7*; Lanes 4 to 8: *GuBAS* in recombinant plasmid pCA-TobRB7-BAS.

3.3. Induction, selection, and confirmation of transformed G. uralensis hairy roots

The cotyledons and hypocotyls of *G. uralensis* were infected with recombinant *A. rhizogenes* containing *GuBAS* gene, and well-grown hairy roots were obtained. Fig. 5 showed the transgenic and wide *G. uralensis* hairy roots after culturing 10, 20, and 40 d. PCR verification results showed that two bands with a length of 580 bp and 2300 bp were present in the transgenic hairy root samples, while only one band with a length of 580 bp was present in the negative control samples (Fig. 6). Further sequencing and BLAST analysis results showed that three transgenic *G. uralensis* hairy root lines containing *GuBAS* gene were obtained.

3.4. Copy number analysis of GuBAS gene in transgenic G. uralensis hairy roots

Both the melting curve of *GuBAS* and *Lectin* gene were unimodal which suggested that the primers used in real-time PCR were specific. The standard curve of *GuBAS* was as follows: $Y = -3.1005X + 38.171$ ($R^2 = 0.996$), and the standard curve of *Lectin* was as follows: $Y = -3.0537X + 36.164$ ($R^2 = 0.999$). Based on the dates listed in Table 3, copy number of *GuBAS* in the three transgenic hairy root lines was 3, 7, and 4 copies, respectively.

3.5. Assay of GA content in transgenic G. uralensis hairy roots

Fig. 7 showed that the retention time of GA was 15.454 min. The standard curve was established by taking the peak area as X-axis and the mass concentration as Y-axis. The standard curve of GA was as follows: $Y = 1 \times 10^{-6} X + 1.53 \times 10^{-2}$ ($R^2 = 1$). And it had good linearity between 0.2204–4.408 µg. The content of GA in all six samples, three transgenic hairy root lines and three wide hairy root lines, were assayed successfully. GA was only present in the three transgenic hairy root lines, while GA was absent in the wild hairy roots (Fig. 8). The difference of GA content in the wide hairy roots and the transgenic hairy roots was analyzed by

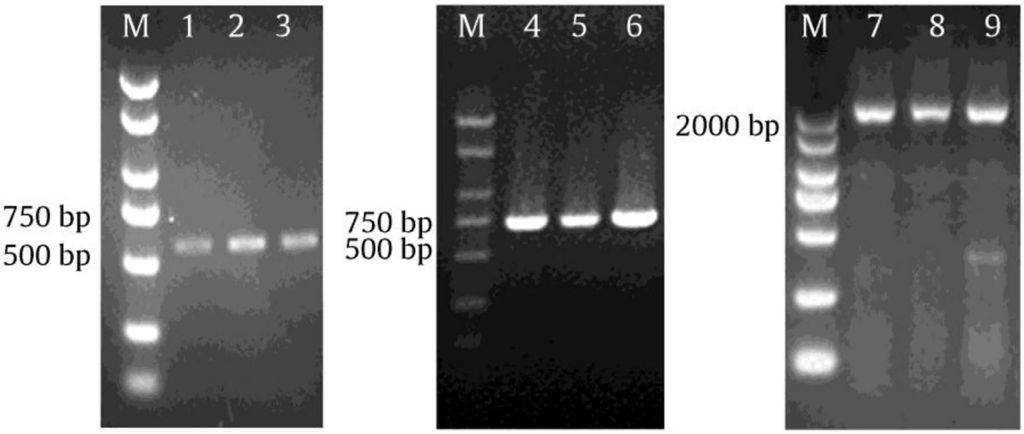

Fig. 4. PCR verification results of recombinant *Agrobacterium rhizogenes* ACCC10060 containing *GuBAS* gene. M: DNA marker; Lanes 1 to 3: *rolC*; Lanes 4 to 6: root-specific promoter *TobRB7*; Lanes 7 to 9: *GuBAS*.

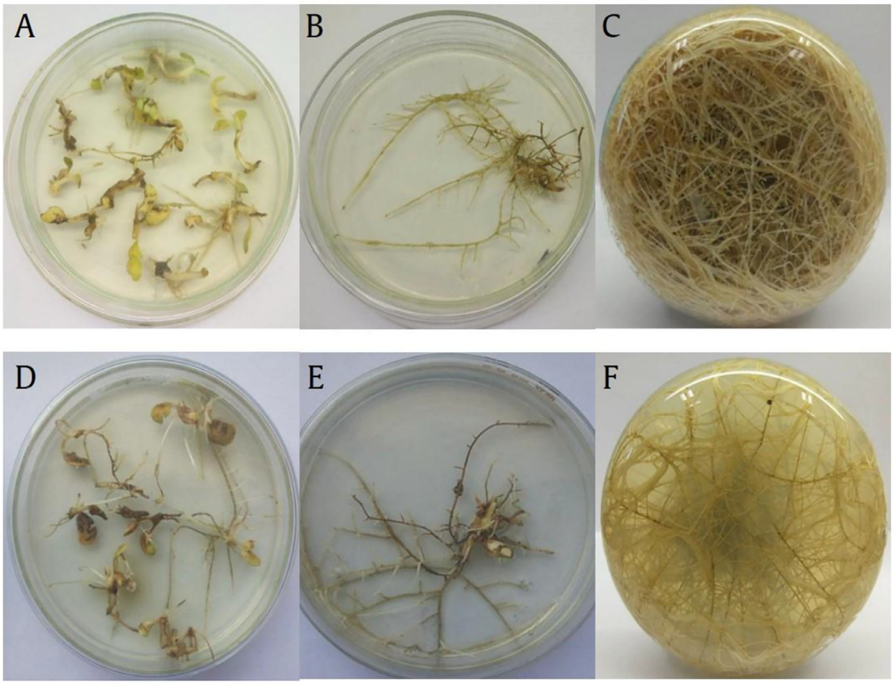

Fig. 5. *Over-expressing root-specific GuBAS gene in transgenic hairy roots and wide hairy roots of G. uralensis*. A, B, and C showed 10-day-old, 20-day-old, and 40-day-old transgenic hairy roots culturing on solid MS medium, respectively. D, E, and F showed 10-day-old, 20-day-old, and 40-day-old wild hairy roots culturing on solid MS medium, respectively.

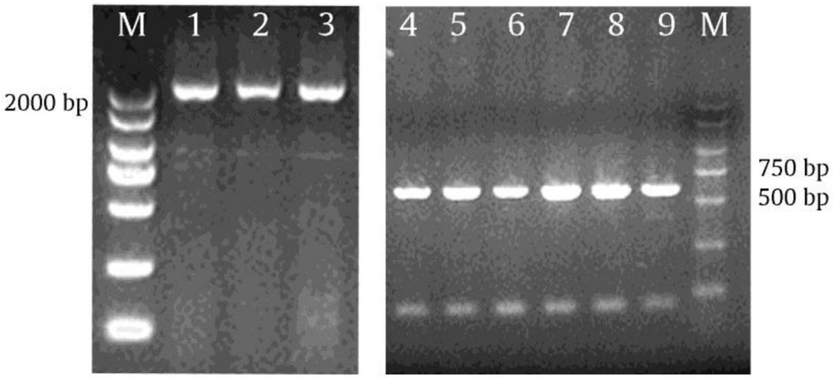

Fig. 6. *PCR verification results of over-expression of root-specific GuBAS gene in transgenic hairy roots and wide hairy roots.* Lanes 1 to 3 showed *GuBAS* in transgenic hairy roots, lanes 4 to 6 showed *rol*C in transgenic hairy roots, and lanes 7 to 9 showed *rol*C in wild hairy roots.

nonparametric test, and the results showed that Z value was -3.7753 and P value was lower than 0.0001, which indicated that great difference existed in the GA content of the wide hairy roots and the transgenic hairy roots. Therefore, over-expression of *GuBAS* in *G. uralensis* hairy roots obviously enhanced the accumulation of GA.

4. Discussion

In this study, we constructed a recombinant plasmid, pCA-TobRB7-BAS, over-expressing root-specific *GuBAS* gene. Three wild and three transgenic hairy root lines of *G. uralensis* were obtained. Using Real-time PCR, the copy number of *GuBAS* in the

Table 3
Copy number of *GuBAS* in different recombinant hairy roots.

No. of lines	Genes	*Ct* value for reaction			*X* value in standard curve			*GuBAS* gene	
		1	2	3	1	2	3	Calculation results	Copy number
B1	*Lectin*	26.33	26.32	26.33	3.22	3.22	3.22	2.52	3
	BAS	27.03	26.86	26.94	3.59	3.65	3.62		
B2	*Lectin*	25.47	25.50	25.49	3.50	3.49	3.50	6.55	7
	BAS	24.47	25.12	24.84	4.42	4.21	4.30		
B3	*Lectin*	25.32	25.32	25.32	3.55	3.55	3.55	3.69	4
	BAS	25.40	25.45	25.37	4.12	4.10	4.13		

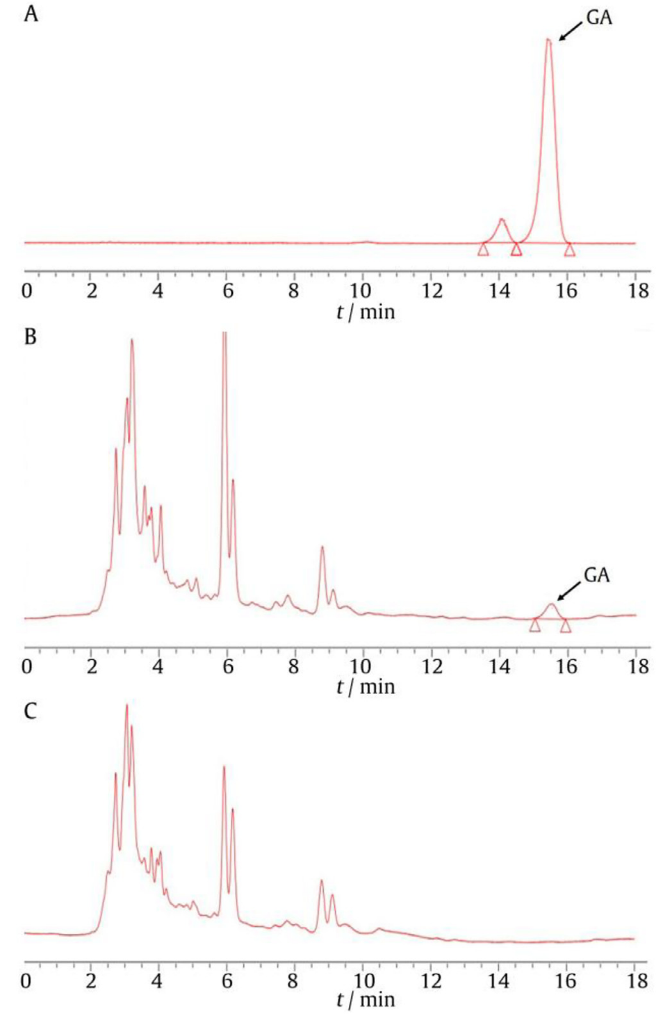

Fig. 7. HPLC of reference substance (A), wild hairy root samples (B), and transgenic hairy root samples (C).

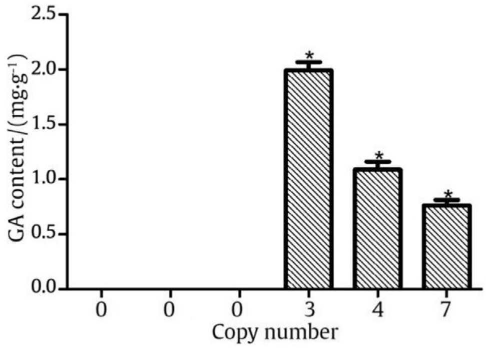

Fig. 8. *Content of GA in hairy roots.* *X*-axis showed copy number of *GuBAS* in different hairy root lines, and *Y*-axis showed content of GA. Nonparametric test was applied to calculate significant difference between content of GA in wild and transgenic hairy roots, and one asterisk above columns indicates that difference was significant ($P < 0.05$).

three transgenic hairy root lines was determined as 3, 4, and 7, respectively. And the HPLC analysis showed that the GA content was significantly higher in all transgenic hairy roots than in the wild ones. Therefore, we believed that over-expressing *GuBAS* root-specifically in hairy roots of *G. uralensis* significantly enhanced GA accumulation. However, our study could not directly contribute to the enhancement of GA in *G. uralensis*, it was a basis for the investigation of GA biosynthetic pathway, which contributed to the cultivation of transgenic *G. uralensis* over-expressing *GuBAS* gene. Therefore, the study is important for further investigations concerned with enhancing the GA content in *G. uralensis*, and revealing the functions of β-*AS* gene in other higher plants.

β-*AS* gene has already been cloned in many plants, such as *Aster sedifolius* L. (Maria et al., 2008), *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim (Long, Li, Yang, & Xing, 2015), *Glycine max* (L.) Merr. (Ali et al., 2016), *Psammosilene tunicoides* W. C. Wu et C. Y. Wu (Liu, Xie, Chen and Qian, 2014), and so on. Chen cloned a 2289bp β-*AS* full-length cDNA sequence from *G. uralensis* and which was expressed in *Saccharomyces cerevisiae* (Chen, Liu, & Zhang, 2013). 392 mRNA sequences, 940 DNA sequences, and 908 amino acid sequences of β-*AS* in higher plants have been registered in NCBI database in Dec, 2017. β-*AS* gene plays a crucial role in controlling and regulating triterpene saponin biosynthesis. RNA interference was used to analyze the function of β-*AS* involved in ginsenoside biosynthesis, it was found that down-regulation of β-*AS* expression resulted in reducing levels of β-amyrin and oleanane-type ginsenoside (Zhao et al., 2015). Expression of β-*AS* gene from *Polygala tenuifolia* Willd. (*PtBS*) in the triterpenoid synthase-deficient yeast mutant GIL77 led to the production of β-amyrin as the sole product (Jin et al., 2014). Also, co-expression of β-*AS* and squalene synthase (*SQS*) genes enhanced the accumulation of β-amyrin in *S. cerevisiae* (Liu et al., 2014), but β-*AS* gene in *G. uralensis* hairy roots had not been reported yet. Hence, it was novel to combine β-*AS* gene with hairy roots to investigate the mechanism of GA biosynthesis and enhancement of GA content.

In our previous studies, we over-expressed squalene synthase (*GuSQS*1) and 3-hydroxy-3-methyliuglutary CoA reductase (*GuHMGR*) gene in *Pichia pastoris* GS115, and found that with the increase of copy number of *GuSQS*1 and *GuHMGR* genes, the content of ergosterol showed an increasing-decreasing-increasing pattern, which proved that copy number variations (CNVs) of functional genes influenced the secondary metabolite production via mevalonic acid (MVA) pathway (Liu et al., 2015). At the same time, we investigated whether over expression *HMGR* in hairy roots could enhance the GA content, but the results were not good, since no GA content was assayed from hairy roots. We speculated that the downstream gene *GuBAS* owned greater impact on the GA content, while upstream gene *HMGR* had less effect on GA content. In this paper, in the three transgenic hairy root lines, it had already been demonstrated that overexpression *GuBAS* enhanced the GA content. Also, it was found that with the increasing of the copy number of *GuBAS*, the GA content was decreased. However, depending on the limits of sample size, whether there is an inverse relationship between the copy number of *GuBAS* and GA content remains to be verified.

Many researchers reported that transgenic hairy roots increased the accumulation of secondary metabolites. Over-expressing *GuSQS*1 gene in *G. uralensis* hairy roots enhanced the GA accumulation (Lu et al., 2008). Over-expressing of *α-L*-rhamnosidase gene (*BbRha*) selectively enhanced the production of ginsenoside Rg_1 in *Panax ginseng* C. A. Meyer hairy roots (Zhang et al., 2015). The amount of catharanthine was increased through the over-expression of *G10H* or both *G10H* and *ORCA3* genes in *Catharanthus roseus* L. G. Don hairy roots (Wang, Hua, Gao, & Zhang, 2010). At the same time, elicitor treatment is also a common strategy for improving the production of secondary metabolites in hairy roots. The total flavonoids production was remarkably enhanced in the *CHI*-transformed hairy roots after being treated with the mixture of Yeast Extract (YE) and Polyethylene glycol 8000 (PEG8000) (Zhang, Liu, Lu, & Gao, 2009). Therefore, different elicitor treatments for enhancing the accumulation of GA content are the next step that we consider performing.

Although we have determined that over-expression of *GuBAS* enhances the accumulation of GA in hairy roots of *G. uralensis*, the molecular mechanism still requires investigation. Zhang reported that over-expression of *SmMYB9b* in *Salvia miltiorrhiza* Bunge (Danshen in Chinese) hairy roots enhanced tanshinone concentration, and KEGG pathway enrichment analysis with de novo RNA-seq data indicated that terpenoid transduction pathways were significantly enriched, transcription of terpenoid biosynthetic genes *SmDXS*2, *SmDXR*, *SmGGPPS*, and *SmKSL*1 were significantly upregulated in *S. miltiorrhiza* hairy roots over expressing *SmMYB9b* (Zhang et al., 2017). Also, the transcriptomic analysis illustrated overexpressing anthranilate synthase (AS) stimulated the overall stress response and affected the metabolic networks in *Catharanthus roseus* hairy roots (Sun, Manmathan, Cheng, & Peebles, 2016). Therefore, many further studies should to be performed to reveal the molecular mechanism of GA biosynthesis in hairy roots of *G. uralensis*.

Conflict of interest

All authors declare that they have no competing interests.

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