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ORIGINAL RESEARCH PAPER

Enhancement of ginsenoside Rg_1 in *Panax ginseng* hairy root by overexpressing the α-L-rhamnosidase gene from *Bifidobacterium breve*

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Abstract
Objectives To improve the production of ginsenoside Rg_1 in *Panax ginseng*.
Results The α-L-rhamnosidase gene from *Bifidobacterium breve* (*BbRha*) was overexpressed into hairy root culture system using *Agrobacterium rhizogenes* A4. Ginsenoside Rg_1 in hairy roots was obtained following transformation via overexpressed gene representing 2.2-fold higher than those of control lines. Several overexpression transgenic hairy root lines were obtained exhibiting markedly increased levels of the corresponding α-L-rhamnosidase enzymatic activity relative to control. Ginsenoside Rg_1 levels in the transgenic lines were higher (2.2-fold) than those of control after following 30 days culturing, while ginsenoside Re contents in tested transgenic lines were found to be lower. The transgenic hairy roots harboring α-L-rhamnosidase gene improved the accumulation of ginsenoside Rg_1 up to 3.6 mg g^{-1} dry weight.
Conclusion *BbRha* gene selectively enhances the production of ginsenoside Rg_1 in *P. ginseng* hairy roots.

Keywords *Bifidobacterium breve* · Ginsenoside Rg_1 · Hairy roots · *Panax ginseng* · α-L-Rhamnosidase · Transgenic hairy roots

Introduction

The roots of ginseng (*Panax ginseng* C.A. Meyer) are used as a traditional medicine in many countries. Ginsenosides, the main bioactive constituents of ginseng, have multiple biological and pharmaceutical applications, related to neuroprotection, anticancer, and antitumor activities (Murthy et al. 2014). The de-glycosylated ginsenosides have greater pharmacological activity than glycosylated ginsenosides due to their relatively higher absorbtion into the bloodstream and functional activities (Hasegawa et al. 2000). Ginsenoside Rg1, a de-glycosylated product of Re, exhibits important pharmacological effects including anti-inflammatory, anti-depressant, and memory-enhancement activities (Song et al. 2013). Ginsenoside Rg1 could be of value in developing new drugs to treat some serious illnesses.

Ginsenoside Re, a major constituent of ginseng, contains a rhamnose residue at the C-6 position of the Rg1. After oral intake, some ginsenoside Re is converted to de-glycosylated metabolites such as

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ginsenosides Rg_1, Rg_2, Rh_1, F_1, and protopanaxatriol (Bae et al. 2005). Rg_1 and Rh_1 are produced from Re, Rg_2, and Rf by glycosyl hydrolases, such as β-glucosidase and α-L-rhamnosidase (Oh et al. 2014; Yu et al. 2002). We have shown that the α-L-rhamnosidase from *Bifidobacterium breve* hydrolyses both the α-1,2 and α-1,6 glucosidic linkages of L-rhamnose residue (Zhang et al. 2015).

Hairy roots, induced by transformation of plant tissue using *Agrobacterium rhizogenes*, have many advantages and high secondary metabolite productivity (Georgiev et al. 2012). Hairy roots with heterologous genes are widely used to enhance the production of secondary metabolites that are rare or undetectable in mother plants (Gandhi et al. 2015; Georgiev et al. 2012; Mehrotra et al. 2013). For example, *Catharanthus roseus* hairy roots have been transformed using bacterial halogenase genes. Transfer of two of these halogenases, *PyrH* and *RebH*, which chlorinate the indole ring of tryptophan, produced different halogenated metabolites (Runguphan et al. 2010; Yeh et al. 2005; Zehner et al. 2005).

In this study, the α-L-rhamnosidase gene from *B. breve* (*BbRha*) was introduced into ginseng roots to produce the transgenic hairy roots successfully. The ginsenoside Rg_1 increased obviously in the hairy root compared to the control hairy roots. Thus, it might be a potential method in increasing the yield of ginsenoside Rg_1 from ginseng.

Materials and methods

Bacterial strains, plasmid and culture conditions

The recombinant vector pET28a-*BbRha* harboring *BbRha* gene from *B. breve* ATCC 15700 (GenBank accession number, CP006715.1) was constructed as previously described (Zhang et al. 2015). *E. coli* (DH5α) and *Agrobacterium rhizogenes* (A4) were employed for gene cloning and transformation, respectively, with the strains grown in lysogeny broth (LB). The plasmid pBI121 was used as plant expression vector.

Vector construction and plant transformation

The amplified PCR product from pET28a-*BbRha* with restriction sites was digested with *Bam*HI and *Sac*I. The fragment was then inserted to the corresponding site of the pBI121 vector. The recombinant binary vector pBI121 with open reading frame integrity of the inserted DNA was confirmed by DNA sequence analysis. The construct was introduced into *A. rhizogenes* A4 by an electroporation method, and subsequently, it was utilized for the transformation of ginseng.

Ginseng (*Panax ginseng* C. A. Meyer) was collected from Fusong County, Jinlin Province, China. The 4-year-old fresh ginseng roots were used for hairy roots induction. Ginseng roots were sterilized and cultured as described previously (Thanh et al. 2005). The pBI121-*BbRha* and pBI121 (control) vectors were transformed into *P. ginseng* using *A. rhizogenes* A4 strain as described previously (Mehrotra et al. 2013). The transformed hairy roots were identified using RT-PCR to check the *BbRha* and *Rol* genes. The successfully transformed hairy roots were transferred to 1/2 MS medium.

Analysis of transgenic hairy roots

Total RNA from hairy roots was extracted using Trizol and treated with DNase I to remove residual DNA, and then reverse-transcribed into cDNA. mRNA expression levels of *BbRha* and *Rol* genes were quantified by RT-PCR and quantitative real-time PCR (qRT-PCR) analysis according to the manufacturer's instructions. All the RT-PCR products were assayed by electrophoresis. qRT-PCR was analyzed according to the method as previously described (Schmittgen and Livak 2008), and the data have been shown after normalization with the *β-actin* (GenBank accession number AY907207) internal control. All the PCR reactions were performed in triplicate. The primers used in this study are listed in Supplementary Table 1.

Assay of α-L-rhamnosidase activity in hairy roots

The hairy roots were harvested and rinsed with sterilized double-distilled water at room temperature and then blotted-dried on filter paper. Approximately 1 g hairy roots were ground into a fine powder in liquid N_2 and re-suspended in 1 ml buffer A (pH 6.5, 50 mM Tris/HCl, 5 mM DTT, 1 % polyvinylpolypirrolidone, and 40 mM ascorbic acid). After vortexing for 30 s, each sample was stored on ice before centrifugation at 15,000×*g* for 20 min at 4 °C to remove debris. Supernatants were transferred into a

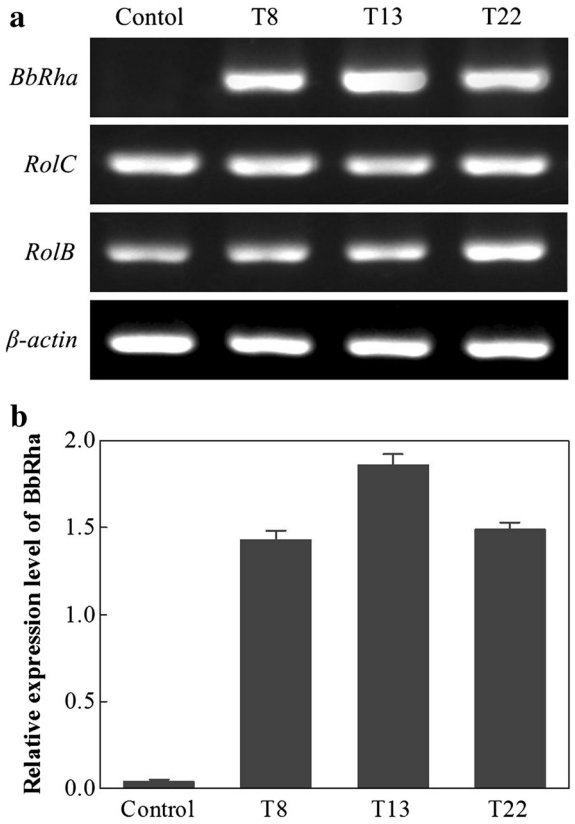

Fig. 1 RT-PCR and qRT-PCR analysis of *BbRha* transcripts in control (empty vector) and *BbRha*-overexpressing (T8, T13, and T22) hairy root lines harvested at 15 day after subculture of the culture cycle. The *error bars* represent the standard error of means of three independent replicates. **a** RT-PCR. **b** qRT-PCR

new centrifuge tube and volumes were adjusted to 2 ml with cold buffer A. The protein concentration was determined using Pierce BCA Protein Assay Kit following the manufacturer's protocol. α-L-Rhamnosidase activity of the hairy roots was determined by measuring the amount of *p*-nitrophenol (*p*NP) transformed from *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NP-Rha, 10 mM) prepared with buffer A at pH 6.5. The final mixture in a microcentrifuge tube contained 200 µl hairy root extract and 200 µl *p*NP-Rha stock was incubated for 2 h at 55 °C (Zhang et al. 2015). Following incubation, the reaction was stopped using an equal volume of 200 mM Na_2CO_3. The released *p*NP was determined at 405 nm. One unit of the enzyme activity (U) was defined as the amount of enzyme releasing 1 µmol *p*NP from the substrate per min (De Winter et al. 2013). All the assays were performed in triplicate.

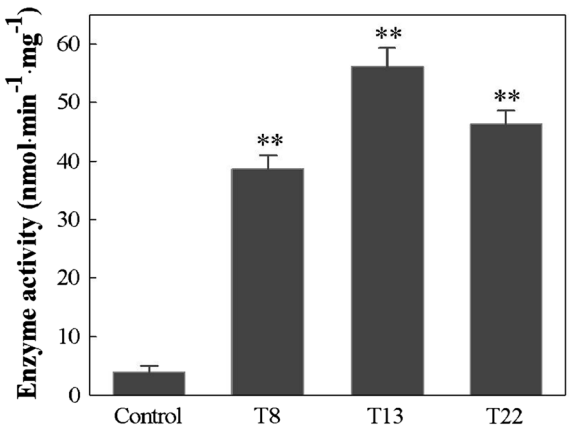

Fig. 2 Comparison of α-L-rhamnosidase activity in control line with overexpression lines (T8, T13, and T22) harvested at day 20. The control line is a hairy root transformed by *Agrobacterium* harboring the empty vector. The enzyme activity of extracts was defined as nmol of ginsenoside Re incorporated into Rg_1 per min of 1 mg protein. Reaction mixture contained 200 µl of hairy root extract and 200 µl of ginsenoside Re (final concentration 1 mM) stock in a microcentrifuge tube and was incubated for 1 h at 55 °C. *Data* represent the means of three independent experiments and *error bars* represent the standard deviation. The differences between the transgenic hairy roots and control hairy roots are statistically significant (*$P < 0.05$, **$P < 0.01$)

Quantitative analysis of ginsenoside Rg_1 using HPLC

Ginsenosides were extracted using 80 % (v/v) methanol and washed with diethyl ether, followed by extraction with n-butanol. The butanol layer was then evaporated to produce a saponin fraction. Each sample was dissolved in methanol for analysis. Ginsenosides were assayed by HPLC at 203 nm, using a C18 column, eluted with water/acetonitrile as the mobile phase at 9:1 to 1:1 (v/v) for 60 min. All the HPLC analyses were performed in triplicate.

Results and discussion

Establishment of hairy root overexpressing *BbRha*

Explants of ginseng roots were transformed with *A. rhizogenes* A4 harboring the pBI121-*BbRha* and pBI121 (control) binary vector respectively. Thirty-six independent transgenic lines were established through selection on kanamycin. Kanamycin-resistant hairy roots were confirmed by RT-PCR and qRT-PCR

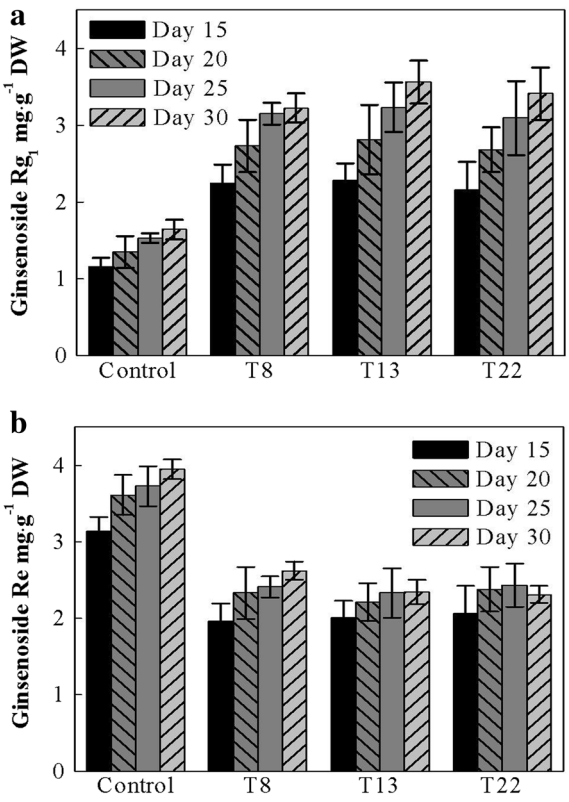

Fig. 3 Analysis of ginsenoside Rg_1 (**a**) and Re (**b**) in control line and overexpression lines at days 15, 20, 25, and 30 of the culture cycle. *Data* represent the means of three independent experiments and *error bars* represent the standard deviation

analysis. Three positive *BbRha*-overexpressing hairy root lines (T8, T13, and T22) were obtained after kanamycin screening and PCR assaying (Fig. 1). One of the six hairy root lines containing the empty vector and evidencing the same level was ultimately selected as control (data not shown). The expression level of *BbRha*-overexpressing lines (T8, T13, and T22) tested is much higher than that of control line (Fig. 1). All the hairy root lines were adapted to liquid 1/2 MS medium and maintained for six generations before further analysis. The expression levels of *BbRha* in the transgenic hairy root lines were further verified using qRT-PCR again. The results of qRT-PCR analysis revealed that *BbRha* gene overexpression induced the accumulation of its transcription in the *P. ginseng* hairy roots. *BbRha* was strongly overexpressed in *BbRha*-overexpressing hairy root lines with the relative expression levels of 36-, 46.5- and 37-fold more compared to the control hairy root lines (Fig. 1b). Consistent with these findings, the lines also

Table 1 Substrate specificity of *B. breve* α-L-rhamnosidase

Substate	Rhamnose released from the total (%)		
	Enzyme (R)	Enzyme (T13)	Enzyme (control)
Rutin	97.4	82.6	6.2
Hesperidin	72.1	68.1	6.8
Naringin	35.2	33.2	5.8
Ginsenoside Re	39.6	36.1	5.3

R recombinant α-L-rhamnosidase from *E. coli* BL21 (56 U protein mg^{-1}, 1.1 mg protein ml^{-1}, and 1.2 U enzyme ml^{-1} final concentration). T13: α-L-rhamnosidase from the hairy roots of T13 line (4.8 U protein mg^{-1}, 0.5 mg protein ml^{-1}, and 1.2 U enzyme ml^{-1} final concentration). Control: α-L-rhamnosidase from the hairy roots of control line (the same protein concentration as T13). Substrate concentration: 5 mM rutin, hesperidin, naringin, and ginsenoside Re. The reaction was performed in sodium phosphate buffer (pH 6.5), 5 % (v/v) DMSO, and at 55 °C for 120 min

evidenced an increased level of α-L-rhamnosidase activity when compared to the controls (Fig. 2). The α-L-rhamnosidase activity in the T8, T13, and T22 hairy roots was 10-, 14.5-, and 12-fold higher than that of the control. Therefore, these results indicate that the *BbRha*-overexpressing hairy root lines were generated successfully, and they enhanced the activity of α-L-rhamnosidase in transgenic hairy roots of ginseng.

Effect of *BbRha* on the ginsenoside Rg_1 levels

To analyze the effect of *BbRha* on the ginsenoside biosynthesis gene expression in ginseng hairy roots, the genes, *SQS* (squalene synthase), *SE* (squalene epoxidase), and *DDS* (dammarenediol II synthase) were analyzed by qRT-PCR. However, the expression level of ginsenoside biosynthetic genes (*SQS*, *SE*, and *DDS*) analyzed in this study was unchanged in *BbRha*-overexpressing hairy root lines (data not shown). To investigate the effect of *BbRha* on the levels of ginsenoside Rg_1, the *BbRha*-overexpressing hairy roots were analyzed by HPLC. High levels of ginsenoside Rg_1 were detected in the hairy roots lines expressing *BbRha*, compared to the control hairy roots. *BbRha*-overexpressing hairy root lines produced 1.9–2.2 times more ginsenoside Rg_1 (2.16–3.56 mg g^{-1} DW) than that of the control hairy root line (1.16–1.64 mg g^{-1} DW) (Fig. 3a). On the other hand, ginsenoside Re contents in tested *BbRha*-overexpressing lines (1.96–2.62 mg·g^{-1} DW) were lower than those of control lines (3.14–3.95 mg g^{-1} DW) (Fig. 3b).

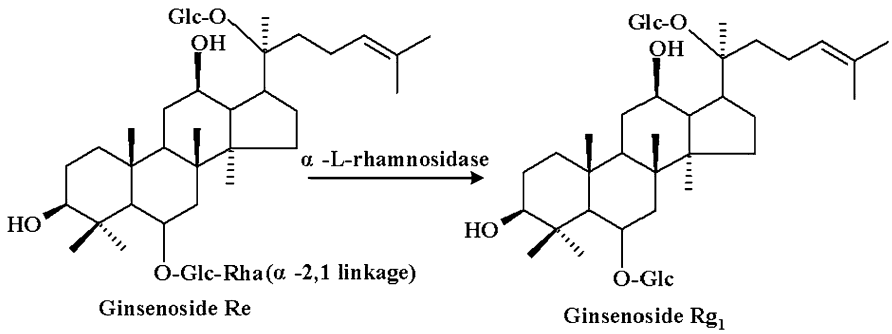

Fig. 4 The proposed mechanism of ginsenoside Rg_1 enhancement by α-L-rhamnosidase

BbRha-overexpressing lines of *P. ginseng* did not show any differences in terms of ginsenosides except Re and Rg_1 when compared to the controls. α-L-Rhamnosidase is known to be an inverting enzyme, being able to catalyze transglycosylation reactions from a large number of natural glycosides containing terminal α-L-rhamnose (De Winter et al. 2013). α-L-Rhamnosidase can be used in the preparation of many drugs and drug precursors. It hydrolyses diosgene (a saponin) to produce α-L-rhamnose and diosgenin used in the synthesis of clinically useful steroid drugs such as progesterone (Elujoba and Hardman 1987). A ginsenoside-α-L-Rhamnosidase was purified and characterized from *Absidia sp*.39 (FFCDL-39) strain. The enzyme hydrolyzed the C-6, α-L-rhamnoside of ginsenoside Rg_2 to produce ginsenoside Rh_1 (Yu et al. 2002). Similarly, the structure of ginsenoside Re contains a terminal α-L-rhamnose at C-6 position of Rg_1. Therefore, we can presume that *BbRha* may participate in the hydrolysis of rhamnose residue from the ginsenoside Re.

Mechanism of enhancement ginsenoside Rg_1 in ginseng hairy roots overexpressing *BbRha*

To explore the mechanism of the ginsenoside Rg_1 enhancement by *BbRha* gene in ginseng hairy roots, recombinant α-L-rhamnosidase was prepared using *E. coli* BL21 (Zhang et al. 2015), and the substrate specificity of the α-L-rhamnosidase was identified using three substrates rutin (α-1,6 linkage), hesperidin (α-1,6 linkage), naringin (α-1,2 linkage), and ginsenoside Re (α-1,2 linkage). As shown in Table 1, α-L-rhamnosidase from recombinant *E. coli* and overexpressing hairy roots hydrolysed both the α-1,2 and α-1,6 glucosidic linkages. However, it displayed broad substrate specificity that may allow its application in the hydrolysis of other rhamnoside moieties. Based on the above results, it could be inferred that the ginsenoside Rg_1 accumulation ascribing this enzyme is able to hydrolyze α-1,2 linkage to β-D-glucoside towards ginsenoside Re in the transgenic ginseng hairy roots. The proposed bioconversion pathway of Re by α-L-rhamnosidase in transgenic ginseng hairy root is shown in Fig. 4.

In summary

BbRha gene was introduced into ginseng roots by using *Agrobacterium* A4 to produce overexpressing-*BbRha* ginseng hairy root lines. Although *BbRha* resulted in significant enhancement of ginsenoside Rg_1 levels specifically in transgenic ginseng hairy roots, a more effective engineered metabolism is needed to identify the key enzmes of ginsenosides biosynthetic metabolism from ginseng, and to ensure their appropriate specificity in ginseng species.

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Supporting information Supplementary Table 1: PCR amplification primers of *BbRha, Rol,* and *β-actin* genes.

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