Title:

**Functional Regulation of ginsenoside biosynthesis by RNA interferences of a
UDP-glycosyltransferase gene in *Panax ginseng* and *Panax quinquefolius***

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Abstract

Panax ginseng (Asian ginseng) and *Panax quinquefolius* (American ginseng) have been used as medicinal and functional herbal remedies worldwide. Different properties of *P. ginseng* and *P. quinquefolius* were confirmed not only in clinical findings, but also at cellular and molecular levels. The major pharmacological ingredients of *P. ginseng* and *P. quinquefolius* are the triterpene saponins known as ginsenosides. The *P. ginseng* roots contain a higher ratio of ginsenoside Rg1:Rb1 than that in *P. quinquefolius*. In ginseng plants, various ginsenosides are synthesized via three key reactions: cyclization, hydroxylation and glycosylation. To date, several genes including dammarenediol synthase (DS), protopanaxadiol synthase and protopanaxatriol synthase have been isolated in *P. ginseng* and *P. quinquefolius*. Although some glycosyltransferase genes have been isolated and identified association with ginsenoside synthesis in *P. ginseng*, little is known about the glycosylation mechanism in *P. quinquefolius*. In this paper, we cloned and identified a UDP-glycosyltransferase gene named *Pq3-O-UGT2* from *P. quinquefolius* (GenBank accession No. KR106207). *In vitro* enzymatic activity experiments biochemically confirmed that *Pq3-O-UGT2* catalyzed the glycosylation of Rh2 and F2 to produce Rg3 and Rd, and the chemical structure of the products were confirmed using high performance liquid chromatography electrospray ionization mass spectrometry (HPLC/ESI-MS). High sequence similarity between *Pq3-O-UGT2* and *PgUGT94Q2* indicated a close evolutionary relationship between *P. ginseng* and *P. quinquefolius*. Moreover, we established both *P. ginseng* and *P. quinquefolius* RNAi transgenic roots lines. RNA interference of *Pq3-O-UGT2* and *PgUGT94Q2* led to reduce levels of ginsenoside Rd, protopanaxadiol-type and total ginsenosides. Expression of key genes including protopanaxadiol and protopanaxatriol synthases was up-regulated in RNAi lines, while expression of dammarenediol synthase gene was not obviously increased. These results revealed that *P. quinquefolius* was more sensitive to the RNAi of *Pq3-O-UGT2* and *PgUGT94Q2* when compared with *P. ginseng*.

Keywords: *Panax ginseng*; *Panax quinquefolius*; UDP-glycosyltransferase; RNA

1 interference

2

1 Introduction

2
3 Asian ginseng (*Panax ginseng*) and American ginseng (*Panax quinquefolius*)
4 belong to the genus *Panax* of the *Araliaceae* family. Although it is native to the
5 eastern temperate forest areas of North America, *P. quinquefolius* is currently being
6 cultivated in Australia, China, Japan, Korea and Russia as one of the most extensively
7 used medicinal plants (Cruse-Sanders and Hamrick, 2004; Sun et al., 2010a). The
8 main compounds accounting for the pharmacological features of *P. ginseng* and *P.*
9 *quinquefolius* are ginsenosides, a group of glycosylated triterpene saponins. Various
10 studies have demonstrated that ginsenosides have many beneficial bioactive
11 properties including anti-aging, anti-stress, enhancing physical performance and
12 immune-modulating effects (Schlag and McIntosh, 2013). Until now, more than 80
13 ginsenosides have been isolated from *P. ginseng* and 60 ginsenosides have been
14 identified in *P. quinquefolius* (Chen et al., 2008). Dammarane-type saponins which
15 account for more than 85% of total ginsenosides in *P. ginseng* and *P. quinquefolius*,
16 are classified into protopanaxadiol (PPD) or protopanaxatriol (PPT) type saponins
17 based on their aglycones structures. Modern molecular and biochemical studies have
18 found that PPD-type ginsenosides have biological activities which are opposite of
19 those in the PPT-type ginsenosides. Subsequent studies also have confirmed radically
20 different pharmacological activities between *P. ginseng* and *P. quinquefolius* roots,
21 which is because extracts of *P. ginseng* roots had relatively higher Rg1 (PPT group):
22 Rb1 (PPD group) ratios than *P. quinquefolius* (Schlag and McIntosh, 2013). Although
23 differences in the biological activities of individual ginsenosides have been associated
24 with differences between the ginsenoside compositions of *Panax* plants roots,
25 ginsenoside Rg1, Rb1, Rc, Rd, and Re are the major ginsenosides that commonly
26 occur in both *P. ginseng* and *P. quinquefolius* (Chen et al., 2008).

27 The current ideas of biosynthetic studies have suggested that ginsenosides are
28 mainly biosynthesized utilizing the precursor IPP through the mevalonic acid (MVA)
29 pathway in the cytoplasm and the methylerythritol phosphate (MEP) pathway in
30 plastids (Zhao et al., 2014). 2, 3-oxidosqualene was converted into dammarenediol-II

1 under the action of dammarenediol synthase (*PqDS*) (Fig. 1). β -amyrin synthase
2 (*Pq β -AS*) converts 2, 3-oxidosqualene into β -amyrin and leads to the production of
3 oleanane-type ginsenosides. The hydroxylation steps of dammarenediol-II include:
4 *PqDI2H* produces protopanaxadiol through the hydroxylation of dammarenediol-II at
5 C-12 position in the biosynthesis of dammarane-type ginsenosides. *CYP6H* converts
6 protopanaxadiol to producing protopanaxatriol through the hydroxylation of C-6 and
7 leads to the formation of protopanaxatriol group ginsenosides (Liang and Zhao, 2008).
8 Both cytochrome P450 enzymes (P450s) and UDP-dependent glycosyltransferases
9 (UGTs) are supergene families and they play key roles in the structural diversity of
10 triterpenoid saponins (Seki et al., 2015). Benefiting from the advance isolation and
11 analysis technologies, the genes which encode β -amyrin synthase, dammarenediol
12 synthase, protopanaxadiol synthase and protopanaxatriol synthase have been cloned
13 and identified from both the *P. ginseng* and *P. quinquefolius* (Kim et al., 2015).
14 Moreover, some candidate glycosyltransferases genes involved in ginsenoside
15 biosynthesis have been revealed and identified by several previous *P. ginseng* and *P.*
16 *quinquefolius* transcriptome studies (Li et al., 2013; Sun et al., 2010b). As for *P.*
17 *ginseng*, five UDP-glycosyltransferases have been identified. *PgUGT74AE2* could
18 transfer a glucose moiety from UDP-glucose to the C3 hydroxyl groups of
19 protopanaxadiol and compound K to yield ginsenoside Rh2 and F2, respectively;
20 *PgUGT94Q2* could transfer a glucose moiety from UDP-Glc to Rh2 and F2 to form
21 Rg3 and Rd, respectively (Jung et al., 2014b). *UGTPg1* could transfer a glucosyl
22 moiety to the C20 hydroxyl groups of dammarenediol-II, protopanaxadiol, Rh2, Rg3
23 and protopanaxatriol to produce 20S-O- β -(D-glucosyl)-dammarenediol II (DMG),
24 compound K, F2, Rd and F1, respectively (Yan et al., 2014). *UGTPg101* not only has
25 the capability to transfer a glucosyl moiety to the free C20-OH of PPT to produce F1
26 but glycosylate the C6-OH of F1 to produce Rg1, subsequently, *UGTPg100* transfers
27 a glucosyl moiety to the C6 hydroxyl groups of PPT and F1 to generate ginsenoside
28 Rh1 and Rg1, respectively (Wei et al., 2015). However, compared with several
29 transcriptome data and plenty of research on *P. ginseng*, much less is known about the
30 biosynthetic pathway of ginsenosides in *P. quinquefolius*.

1 In this study, we cloned and identified a UDP-glycosyltransferases gene
2 (*Pq3-O-UGT2*) encoding enzyme which is involved in the biosynthetic pathway of
3 ginsenosides from *P. quinquefolius*. High identity of amino acid sequences and
4 similarity functions of *Pq3-O-UGT2* and *PgUGT94Q2* suggested a closely
5 evolutionary relationship between *P. ginseng* and *P. quinquefolius*. Furthermore, we
6 constructed RNAi destination vector according to the highly conserved domain of
7 *Pq3-O-UGT2* and *PgUGT94Q2* and established *P. ginseng* and *P. quinquefolius* RNAi
8 hairy root lines. The content of ginsenosides and the expression of relative upstream
9 genes in transgenic hairy root culture system indicated different expression regulatory
10 mechanisms of two *Panax* species. Therefore, this work is helpful in elucidating the
11 mechanism of ginsenosides biosynthesis in *Panax* species and provides important
12 enlightenment for understanding the role of UGTs in plants.

13 14 **1. Materials and methods**

15 16 *1.1. Isolation and sequencing analysis of Pq3-O-UGT2 of P. quinquefolius*

17
18 Total RNA was isolated from the hairy roots of *P. quinquefolius* using the RNA
19 Reagent kit (TaKaRa Dalian China) and the first-strand cDNA was prepared from 1
20 µg of the total RNA using Prime Script™ RT-PCR Kit (TaKaRa Dalian China).
21 Degenerate primers were designed based on the highly conserved Putative Secondary
22 Plant Glycosyltransferase (PSPG) motif after comparing the conserved sequences of
23 *PgUGT94Q2* (JX898530) and *UGTPg29* (KM401911) by BLAST P and the full
24 length sequences were obtained by 5' and 3' rapid-amplification of cDNA ends
25 (RACE) technique. The amplified products were cloned into the pGEM-T vector and
26 sequenced. For the phylogenetic analysis, the deduced amino acid sequences of the
27 glucosyltransferases from other plants were aligned using the CLUSTAL X program
28 and the phylogenetic tree was generated by the neighbor-joining method of the
29 MEGA 5.1 program.

1.2. Expression analysis of *Pq3-O-UGT2* in yeast

To construct an expression plasmid for yeast, we designed primers with the addition of *Sam* I and *Sac* I sites (P1-F: 5'-CCC GGG ATG GAT AAC CAA AAA GGT AGA ATC A-3' and P1-R: 5'-GAG CTC TTG TTC ATC TTT CTT CTT CTT ACA A-3'). The PCR conditions were designed as: 98°C for 30s, then 35 cycles of 98°C for 15s, 54°C for 35s, 72°C for 95s, with a final 10 min extension at 72°C. The PCR product was purified, ligated to pGEM-T vector, sequenced, excised and re-cloned between the *Sam* I and *Sac* I of pAUR123 expression vector (TaKaRa Dalian China). The recombinant expression vector and an empty vector were transformed into *Saccharomyces cerevisiae* strain INVSc1 yeast according to the previous studies with some modifications(Sun et al., 2013; Wang et al., 2014b). Transformed cells were selected using antibiotics aureobasidin A (AbA) after 4 days at 30 °C and then subcultured on YPD liquid medium.

After being cultured in YPD liquid medium for 4 days, the cell pellets were collected, disrupted and used for enzyme assays reaction. 200 µL volume of the reaction mixture consisted of recombinant yeast extract: 100 mM phosphate buffer (pH 7.8), 2% Tween-20, 6 mM UDP-glucose, 0.6 mM acceptor substrate (dissolved in methanol) and the reaction was incubated at 35 °C for 8 h. The enzymatic reaction was stopped with 200 µL n-butanol, evaporated, and re-dissolved with the equal volume of methanol for HPLC and LC–electrospray ionization (ESI) MS analysis.

1.3. HPLC/ESIMS Analysis

To analyze the products extracted from the transformed yeast strains, HPLC analyses were performed on an Agilent 1200 Series 3300 Ion Trap LC system (Agilent, Waldron, Germany) equipped with electrospray ionization (ESI) apparatus. Chromatographic Separations were achieved at 30 °C using a reverse-phase C18 column (5 µm, 4.6×250 mm) and wavelength was detected at 202 nm. The mobile phase system consisted of HPLC grade water containing 0.01% phosphoric acid

(solvent A) and HPLC grade acetonitrile (solvent B). The flow rate was set at 0.8 ml min⁻¹ and the program of mobile phase system was set at: 0-10 min, 55 % A and 45 % B; 10-20 min, 50 % A and 50 % B; 20-40 min, 40 % A and 60 % B; 40-55 min, 25 % A and 75 % B; 55-65 min, 15 % A and 85 % B; 65-70 min, 55 % A and 45 % B. Then, to further determine the products of enzymatic reactions, the extracted products were subsequently analyzed by a triple-quadrupole tandem mass spectrometer. Negative ionization mode was conducted using an ion source voltage of 3.6 kV. The temperatures of ion source gas and desolation gas were set at 150 °C and 350 °C, respectively. The desolation gas flow rate was kept at 0.8 ml min⁻¹ and full scan of ions range from 100 to 1000 molecular weights was acquired using analysis scripstarter software.

1.4. Construction of RNAi silencing vector and RNAi hairy root lines

Sequence analyses of *Pq3-O-UGT2* and *PgUGT94Q2* genes were performed using CLUSTAL X software and a highly conserved domain of two genes nucleotide sequences was selected to construct RNAi silencing component. Two primers (P2-F: 5'-CCC ATC TCA TGC TCC CAC TCA G-3' and P2-R: 5'-GTG GAC CAA CAG GAA CCA AAG T-3') were designed to amplify the region from nucleotide 251 to 676 (426-bp) of *Pq3-O-UGT2*. The PCR-amplified products were sequenced, and cloned into pMD20-T vector (TaKaRa Dalian China) and used as the template for sense and antisense fragment. Primers of sense and antisense fragment were designed with *Sam* I, *Bgl* II and *Sac* I adapters (Sense-F: 5'-TCC CCC GGG CCC ATC TCA TGC TCC CAC TCA G-3', Sense-R: 5'-GGA AGA TCT CCC TTT CTC CAT TGC CTC CAC AGC-3', Antisense-F: 5'-GGA AGA TCT CCC ATC TCA TGC TCC CAC TCA G-3', Antisense-R: 5'-TCC CCG CGG GTG GAC CAA CAG GAA CCA AAG T-3'). Two primers (Sense-F, Antisense-R) were selected to amplify the final RNAi silencing component. The amplified RNAi components were cloned into pGEM-T vector and transferred to the RNAi destination vector pBI121 later by double digestion with *Sam* I and *Sac* I. The final recombined vector was transformed into *Escherichia coli* DH5 α , sequenced, and subsequently transformed into *A. rhizogenes* A4.

Roots of 3-year-old *P. ginseng* and *P. quinquefolius* were provided by Agricultural

Engineering laboratory, Jilin University. Genetic transformation of *P. ginseng* and *P. quinquefolius* was carried out according to the method in our previous report (Wang et al., 2014a; Zhao et al., 2015). After co-cultivation with *A. rhizogenes* A4 suspension for 24h, the root discs were transferred to culture on MS solid medium supplemented with 20 mg/l kanamycin, 3 % sucrose at 25 °C until hairy roots started to appear at the infected sites. Hairy roots transformed by *A. rhizogenes* A4 harboring empty vector were used as controls and the *in vitro* cultured hairy roots derived from independent transgenic *P.quinquefolius* were regarded as an independent sample. The controls of hairy roots were confirmed by amplify the rolC gene with primers (rolC-F: 5'AAG TAT CCA GTT ATT AAA CCA GA3', rolC-R: 5'ATA AAT TTG TGG ACC TAT ACC TA3'). The transgenic hairy roots were selected by kanamycin (20 mg/L) resistance and further confirmed by amplifying a specific fragment with primers (35S-F: 5'-TAG AGG ATC CCC GGG TGG TCA GTC CCT-3', NOS-R: 5'-GAG CTC GGT AGC AAT TCC CGA GGC TGT-3').

1.5. Quantitative PCR analysis

Total RNA was isolated from the transgenic hairy root lines and control groups of *P. ginseng* and *P. quinquefolius*. The nucleotide sequences of these primers were designed as described in Table 2. Quantitative PCR (qPCR) was performed on a Qiagen Rotor Gene Q real-time PCR detector system (Qiagen, Hilden, Germany) using SYBR Green PCR Kit. The PCR reaction mixture (25 µl) contained 1×SYBR Green PCR Master Mix, 0.9 µM each of forward and reverse primers and 0.1 µg of template cDNA. The PCR amplification was performed under the following conditions: 50 °C for 2 min, and 95 °C for 8 min, followed by 40 cycles of 95 °C for 15 s and then 60 °C for 30 s. The relative expression value of each gene was calculated by comparing Ct (cycle threshold) of target gene with the control gene β -actin, in which the $2^{-\Delta\Delta C_t}$ method was used. The qRT-PCR analyses were performed in triplicate, and the sizes of PCR products were determined by electrophoresis in 1% agarose gel.

1.6. Ginsenoside analyses by HPLC

Ginsenosides of both transgenic hairy root lines and control groups of *P. ginseng*

1 and *P. quinquefolius* were extracted and analyzed high-performance liquid
2 chromatography (HPLC) according to the protocol reported in our lab (Sun et al.,
3 2013; Zhao et al., 2015). The HPLC separation was performed on a C18 column (5
4 μm , 4.6 $\times$ 250 mm, Shimadzu, Kyoto, Japan), maintained at 30 °C, with water and
5 acetonitrile as mobile phase. The time and ratios of acetonitrile and water used a
6 protocol previously reported in our lab (Zhao et al., 2015). The flow rate of the mobile
7 phase was set at 1.0 ml min⁻¹, and ginsenosides were monitored at a wavelength of
8 202 nm. Each ginsenoside was compared with the authentic ginsenoside samples
9 purchased from Sigma. Quantitative analysis was calculated from the peak area of the
10 respective compound according to the methods previously reported in our laboratory
11 (Zhao et al., 2015).

13 2. Results

15 2.1. Isolation and analysis of *Pq3-O-UGT2*

17 The degenerate primers and rapid amplification of cDNA ends (RACE) were used
18 to amplify the candidate glycosyltransferase genes from *P. quinquefolius*. A
19 full-length sequence with an open reading frame (ORF) of 1,329-bp long encoding a
20 protein of 469 amino acids with a calculated molecular mass of 51.1 kDa was
21 obtained through reverse transcriptase-PCR (RT-PCR) analysis. After being isolated
22 and purified, this sequence was selected for further analysis and named *Pq3-O-UGT2*
23 (GeneBank accession No. KR106207). The results of online BLASTP search showed
24 that the deduced amino acid sequence of *Pq3-O-UGT2* shared extremely high
25 sequence similarity of 99.55% and 99.32% with that of *P. ginseng*
26 UDP-glucosyltransferases *PgUGT94Q2* and *UGTPg29*; respectively. A phylogenetic
27 tree was constructed to further evaluate the homology and evolution relationship
28 between *Pq3-O-UGT2* and other glycosyltransferase genes (Fig. 2). As expected,
29 phylogenetic analysis also demonstrated that *Pq3-O-UGT2* gene has a close
30 evolutionary relationship with UDP-glucosyltransferases of *P. ginseng*, and was
31 probably involved in glycosylation of PPD-type ginsenoside in *P. quinquefolius*.

33 2.2. Ectopic expression of *Pq3-O-UGT2* cDNA in yeast

The full-length cDNA clone of *Pq3-O-UGT2* was inserted into a yeast expression vector pAUR123 and expressed in INVSc1 *S. cerevisiae* yeast cells for in-vivo biochemical experiments. After being cultured in medium containing antibiotic AbA for 72 h, the yeast extract was collected and fed with UDP-glucose and acceptor substrate. The products of enzymatic reaction were analyzed by high performance liquid chromatography electrospray ionization mass spectrometry (HPLC/ESI-MS). It has been indicated that *PgUGT94Q2* and *UGTPg29* could readily convert Rh2, F2 into Rg3, Rd, respectively. Thus both Rh2 and F2 were fed in the yeast extract to functionally examine the activity of *Pq3-O-UGT2*. For the selected transformations in this study, when incubated with Rh2, *Pq3-O-UGT2* glycosylated the C3-O-glucoside of Rh2 to produce Rg3, as monitored by HPLC (Fig. 3c); when incubated with F2, *Pq3-O-UGT2* glycosylated the C3-O-glucoside of F2 to produce Rd (Fig. 3d). These two signals were not detected from the control yeast containing the empty vector (Fig. 3b). When incubated with both Rh2 and F2, there were four peaks at the retention time of 31.42, 37.51, 41.87 and 60.46 min were all observed from HPLC (Fig. 3e). These results suggest that *Pq3-O-UGT2* is a bifunctional UGT gene. The two signals at 31.42 and 41.87 min were further analyzed using HPLC/ESI-MS (Fig. 4). The HPLC/ESI-MS spectra of a peak at the 41.87 min retention time from enzymatic reaction included the m/z ratios of 783.8 [M-H]⁻, 621.6 [M-Glc-H+H₂O]⁻, 459.6 [M-2Glc-H+H₂O]⁻, which matched with the ion peaks of the authentic ginsenoside Rg3 (Fig. 4a). In addition, The HPLC/ESI-MS spectra of the peak at 41.87 min retention time included the m/z ratios of 945.3 [M-H]⁻, 783.4 [M-Glc-H+H₂O]⁻, 621.5 [M-2Glc-H+H₂O]⁻ and 459.6 [M-3Glc-H+H₂O]⁻, which was the same as those of the authentic ginsenoside Rd (Fig. 4b). These results demonstrated that *Pq3-O-UGT2* was an authentic UDP-glycosyltransferase gene, which could transfer a glucose moiety to the C3-O-glucoside of ginsenoside Rh2 and F2, and convert them to ginsenoside Rg3 and Rd during ginsenoside biosynthesis in *P. quinquefolius*.

2.3. Functional analysis of two UGTs in transgenic hairy roots

To functionally analyze the role of two UGT genes in transgenic *P. ginseng* and *P. quinquefolius* plants, constitutively expressed 3-O-UGT2-RNAi component was generated according to a highly conserved nucleotide domain within two genes. The

1 induction and selection of all transgenic root lines were performed using the protocol
2 described in our previous reports (Sun et al., 2013; Zhao et al., 2015). The hairy root
3 originated from independent root discs of *P. quinquefolius* and *P. ginseng* was
4 considered as an independent sample. After being subcultured for more than five
5 generations in liquid MS medium, the control and RNAi root lines were used for
6 further analysis. Two control root lines and four RNAi lines were randomly selected
7 among all the *P. quinquefolius* and *P. ginseng* root discs samples, respectively. There
8 were no obvious morphological differences observed between the transgenic hairy
9 roots of *P. quinquefolius* and *P. ginseng* compared with the wild type (Fig. 6a).
10 However, when cultured in liquid MS medium, the RNAi lines exhibited a slower
11 growth than control lines in both *P. quinquefolius* and *P. ginseng*. Besides, for the dry
12 weight level of hairy roots, the *P. quinquefolius* samples were observed stronger
13 inhibition of transgenic growth compared with *P. ginseng* (Fig. 6b).

14 The ginsenoside content in all 12 cultured hairy roots was determined by HPLC
15 analysis (Fig. 5). In control lines, the levels of PPD-group (Rb1, Rb2, Rc, Rd, and
16 Rg3) and total ginsenosides were clearly higher in *P. quinquefolius* than in *P. ginseng*
17 (Fig. 5). In all RNAi lines, the Rg3 ginsenoside were nearly absent (Table 1). The
18 content of PPT-group and total ginsenosides was clearly reduced in both *P.*
19 *quinquefolius* and *P. ginseng* transgenic RNAi lines. Yet the levels of PPT-group
20 ginsenosides (Rg1, and Re) showed a slightly reduced in transgenic lines compared
21 with the controls (Table 1). This would indicate that silencing of 3-O-UGT2 gene
22 could not greatly change the content of PPT-group ginsenosides in *Panax* plants.
23 Furthermore, PPD-group ginsenosides (Rb1, Rb2, Rc, and Rd) were shown a more
24 clear reduction in *P. quinquefolius* transgenic RNAi lines compared with those in *P.*
25 *ginseng* (Fig. 5). This result indicates that RNA interference of 3-O-UGT2 gene has
26 more influence on the production of the PPD-group ginsenosides in *P. quinquefolius*
27 than in *P. ginseng*.

28 To further elucidate the mechanism underlying the alteration of ginsenoside level
29 by RNAi in *P. quinquefolius* and *P. ginseng*. Expression profiling of genes related to
30 ginsenoside biosynthesis was analyzed by quantitative PCR (qPCR). The expression
31 of glycosyltransferase gene (*Pq3-O-UGT2*, and *PgUGT94Q2*) was clearly
32 down-regulated in RNAi transgenic lines compared with control hairy root lines (Fig.
33 7a and b). However, the expression of two upstream genes (protopanaxadiol, and
34 protopanaxatriol synthases) was enhanced in transgenic *P. quinquefolius* and *P.*

ginseng hairy roots compared with the wild-type (Fig. 7c and d). The improvement of protopanaxadiol synthases in *P. quinquefolius* (*PqD12H*) showed clearer than that in *P. ginseng* (*CYP716A47*); the improvement of protopanaxatriol synthases in *P. ginseng* (*CYP716A53V2*) showed more obviously than that in *P. quinquefolius* (*Pq-CYP6H*). Moreover, there was no obviously change in the accumulation of dammarenediol synthases (DS) mRNA between transgenic and control hair root lines in both *P. quinquefolius* and *P. ginseng* (Fig. 7c and d).

3. Discussion

The major bioactive constituents of *P. ginseng* (Asian ginseng) and *P. quinquefolius* (American ginseng) are the triterpene saponins known as ginsenosides. Five UGTs that participate in the biosynthesis of dammarane-type ginsenoside have been identified in *P. ginseng* (Jung et al., 2014b; Wang et al., 2015; Wei et al., 2015; Yan et al., 2014). However, little is known about the UGTs participating in ginsenoside biosynthesis in *P. quinquefolius*. In the present study, we isolated and regulated a UDP-glucosyltransferase gene (*Pq3-O-UGT2*) from *P. quinquefolius*. The high similarity of *Pq3-O-UGT2* with *PgUGT94Q2* and *UGTPg29* at amino acid level and biological function in ginsenoside biosynthesis indicated a closely evolution relationship between *P. quinquefolius* and *P. ginseng*. Various studies got increased ginsenoside accumulation by chemical elicitors, overexpression or RNAi of key ginsenoside biosynthesis enzymes in ginseng cell suspension cultures or adventitious roots (Kim et al., 2014; Park et al., 2016; Rahimi et al., 2015; Wang et al., 2014b; Zhao et al., 2015). Moreover, RNAi has been used for the identification or validation of biological function of the targeted transcripts of individual genes in planta (Han et al., 2010; Sun et al., 2013). Many reports indicated the possibility of altering ginsenoside levels by regulating genes encoding enzymes involved in ginsenoside biosynthesis. It has reported that inhibiting the expression of protopanaxadiol synthase can depress the levels of protopanaxadiol and protopanaxatriol. Moreover, they also suggested that the P450 synthase content in *P. quinquefolius* is exceeded than that in *P. ginseng* (Sun et al., 2013). The RNAi-mediated inhibition of β -Amyrin

1 synthase decreased levels of oleanane-type ginsenoside Ro and increased the level of
2 total ginsenosides in *P. ginseng* (Zhao et al., 2015). Silencing of CYP716A53v2
3 mRNA in RNAi transgenic roots resulted in lower levels of protopanaxatriol-group
4 ginsenosides and higher levels of protopanaxadiol-group ginsenosides in *P. ginseng*
5 (Park et al., 2016). RNAi of dammarenediol synthase gene in transgenic *P. ginseng*
6 hairy roots caused silencing of DDS expression which leads to a reduction of
7 ginsenoside production to 84.5% (Han et al., 2006). Meanwhile, our study is also
8 aimed at describing the regulation of glycosyltransferases in both *P. quinquefolius* and
9 *P. ginseng*. To confirm in vivo relationship of *Pq3-O-UGT2* and *PgUGT94Q2* in
10 plants, RNA interference of the conserved sequences of two UGTs were constructed
11 by recombinant RNAi plant destination vector. Ginsenoside analysis by HPLC
12 revealed that RNAi of the conserved sequences in both *P. quinquefolius* and *P.*
13 *ginseng* hairy roots brought about down-regulation of ginsenoside Rd,
14 protopanaxadiol-type ginsenoside and total ginsenosides levels, indicating an
15 important role of *Pq3-O-UGT2* in ginsenoside biosynthesis. It has been shown that
16 the content of total ginsenoside in *P. ginseng* is about 4 % while in *P. quinquefolius* is
17 about 5 % (Chen et al., 2008; Sun et al., 2013). Some studies indicated that the extra
18 1 % content of ginsenoside is the result of dammarane type ginsenoside biosynthesis
19 (Chen et al., 2008; Cruse-Sanders and Hamrick, 2004; Jung et al., 2014a; Popovich
20 and Kitts, 2004). During evolution, *Panax* species select the production of
21 dammarene-type tetracyclic triterpene as important defensive compounds to fight
22 against attack by various pathogens and pests for adaptation to their environments. In
23 general, the PPD-group ginsenosides have pharmaceutical activities that are opposite
24 to those of the PPT-group (Kim et al., 2015; Liang and Zhao, 2008). In the present
25 study, the content of Rd, protopanaxadiol-type and total ginsenosides in *P.*
26 *quinquefolius* hairy roots decreased more significantly than those in the *P. ginseng*
27 hairy roots. Therefore, *P. quinquefolius* are more sensitive to the RNAi of
28 *Pq3-O-UGT2* when compared with *P. ginseng*. The result of ginsenoside analysis by
29 HPLC is consistent with the report that *P. ginseng* had a relatively higher ratio of Rg1
30 to Rb1 content and *P. quinquefolius* had a relatively lower ratio of that (Schlag and

1 McIntosh, 2013; Sun et al., 2013). Besides, RNAi transgenic roots showed a clear
2 decrease in the content of ginsenoside Rd and those lines had no obvious growth
3 difference from controls. The comparison between the growth of control and RNAi
4 root lines indicated that *Pq3-O-UGT2* and *PgUGT94Q2* were not involved in growth
5 of *P. quinquefolius* and *P. ginseng* hairy roots. Therefore, primary metabolism in both
6 *P. quinquefolius* and *P. ginseng* could be independent on *Pq3-O-UGT2* and
7 *PgUGT94Q2*.

8 Later, we use qPCR to investigate the expression of upstream genes in both *P.*
9 *quinquefolius* and *P. ginseng* RNAi transgenic roots. The expression level of
10 dammarenediol synthase gene is not significantly changed in all RNAi lines. This
11 result demonstrates that altering *Pq3-O-UGT2* expression by RNAi does not affect the
12 dammarenediol synthase gene in the ginsenoside biosynthesis pathway. It is likely that
13 the down-regulation expression of *Pq3-O-UGT2* led to an increased expression of
14 other upstream genes (protopanaxadiol synthase and protopanaxatriol synthase). Now
15 that protopanaxadiol-type compound synthesis pathway represents a competitive
16 branch with that of protopanaxatriol-type ginsenoside, it is possible that
17 *Pq3-O-UGT2*-RNAi led to an increased metabolism flux toward protopanaxatriol
18 -type compounds. In transgenic hairy roots, the decreased expression of *Pq3-O-UGT2*
19 gene might be attributed to the high flux of P450 toward protopanaxatriol-group
20 secondary metabolites when protopanaxadiol-type biosynthesis is suppressed. The
21 result of qPCR analysis also suggested that the protopanaxadiol-type UGTs genes
22 content or expression in *P. quinquefolius* exceeds that in *P. ginseng*. Additionally, the
23 results of our study possibly implied that *Pq3-O-UGT2* and *PgUGT94Q2* have
24 different expression profiles in *P. quinquefolius* and *P. ginseng*. These findings
25 indicate that *Pq3-O-UGT2* and *PgUGT94Q2* share the same biochemical function in
26 two *Panax* species, but their specific biological functions were determined by
27 difference spatiotemporal expression patterns and environmental factors.

28 Glycosylation is a prominent modification reaction and is always the last step in the
29 biosynthesis of natural compounds (Li et al., 2014; Pace et al., 2015; Seki et al., 2015).
30 However, as to *P. quinquefolius*, the progress of ginsenoside synthetic pathway seems

1 slower in this respect. Here we cloned and identified a UDP-glucosyltransferase gene,
2 evaluated the roles of *Pq3-O-UGT2* gene in ginsenoside biosynthesis in both *P.*
3 *quinquefolius* and *P. ginseng* by RNAi. The result showed that the RNAi-mediated
4 inhibited the expression of *Pq3-O-UGT2* gene is able to depress the levels of
5 ginsenoside Rd, protopanaxadiol-type ginsenoside. The key genes involved in
6 dammarane-type ginsenoside synthesis were clearly up-regulated including
7 protopanaxadiol and protopanaxatriol synthases, which also suggested that *P.*
8 *quinquefolius* had much sensitivity to the RNAi of *Pq3-O-UGT2* when compared with
9 *P. ginseng*: a finding that may explain the molecular basis for the higher
10 protopanaxatriol-type ginsenoside content in *P. quinquefolius*. These results will be
11 helpful for the elucidation of ginsenoside biosynthesis research in *P. quinquefolius* or
12 its closely related species, such as *P. ginseng* and *P. notoginseng*.

13

Acknowledgments

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21 **Figure Legends**

22

23 **Fig. 1. The proposed biosynthesis pathway of ginsenosides in *P. quinquefolius*.**

24 MVA mevalonic acid, MEP methylerythritol 4-phosphate, IPP isopentenyl
25 diphosphate, β -AS β -amyrin synthase, DS dammarenediol synthase.

26

27 **Fig. 2. Phylogenetic tree constructed based on the deduced amino acid sequences**
28 **of *Pq3-O-UGT2* and other glycosyltransferase genes of plant. *DcsUGT***

29 (XP_017250139.1), *EuGT* (AHX74090.1), *FvsUGT* (XP_004303956.1), *NtUGT*
30 (NP_001313072.1), *NtUGT6* (XP_009587669.1), *CcGT* (CDP16611.1), *NsUGT6-like*

1 (XP_009799026.1), *StUGT6-like* (XP_006353724.1), *PpGT* (XP_007216098.1),
 2 *CrGT* (BAF75901.1), *VvGT6-like* (XP_010648215.1), *EgGT* (KCW88565.1),
 3 *EgGT2-like* (XP_010045071.1), *SlUGT6-like* (XP_004243651.1), *SpUGT6-like*
 4 (XP_015082329.1), *EgGT2-like* (XP_010045032.1), *CrUGT* (BAD29721.1),
 5 *PgUGT1* (AIE12479.1), *Pq20-O-UGT1* (AKI06370.1), *PgUGT29* (AKA44579.1),
 6 *PgUGT94B1* (AGR44632.1), *DcsGT-like* (XP_017249390.1), *BcGT* (AGP25113.1),
 7 *StGT-like* (XP_006361439.1), *NtGT-like* (XP_009604940.1), *NtGT-like2*
 8 (XP_016476450.1), *SpGT-like* (XP_015057251.1), *SlGT-like* (XP_004249996.1),
 9 *CaGT-like* (XP_016549951.1), *Vvg-GT* (XP_002264771.2), *NsGT-like*
 10 (XP_009770958.1), *VvGT-like* (XP_002271587.2), *BcUGT5* (AFK79037.1),
 11 *LcGT-like4* (AHK12770.1), *CaGT-like* (XP_016570714.1), *CsUGT94P1*
 12 (BAO51835.1), *NsGT-like* (XP_009775013.1).

13

14 **Fig. 3. Total ion chromatogram of HPLC/ESIMS analysis of the products of *in***
 15 ***vitro* enzymatic reaction.** (a) LC chromatogram of ginsenoside standards. (b) LC
 16 chromatogram of the extracts from the enzymatic reaction fed without any substrates
 17 as a control. (c) LC chromatogram of the extracts from the enzymatic reaction fed
 18 with ginsenoside Rh2. (d) LC chromatogram of the extracts from the enzymatic
 19 reaction fed with ginsenoside F2. (e) LC chromatogram of the extracts from the
 20 enzymatic reaction fed with ginsenosides Rh2 and F2.

21

22 **Fig. 4. HPLC/ESIMS spectra of peak A, B, C and D detected in the extracts of *in***
 23 ***vitro* enzymatic reaction.** Upper panel shows the MS spectrum of the authentic
 24 ginsenoside Rg3 (peak A) detected at a retention time of 41.87 min and Rd standard
 25 (peak B) detected at a retention time of 31.42 min, respectively. Lower panel shows
 26 the MS spectrum of the products of enzymatic reaction (peak C and D) detected in the
 27 at a retention time of 41.87 min and 31.42 min, respectively. The mass spectrometry
 28 spectrum of the products of Rg3 and Rd were the same as that of the standards.

29

30 **Fig. 5. HPLC analysis of ginsenosides contents in control and RNAi root lines.**

Two control lines and four RNAi lines were selected from *P. ginseng* and *P. quinquefolius*, respectively. Each column represents the mean value with the standard error obtained from three independent experiments. Each *asterisk* above the *error bar* indicates significant differences between transgenic and control samples, determined using Student *t* test (* $p \leq 0.05$).

Fig. 6. Growth status of control and RNAi root lines of *P. ginseng* and *P. quinquefolius*. (A) Representative pictures of hairy roots cultured in MS liquid medium for 4 weeks. Pq, *P. quinquefolius*; Pg, *P. ginseng*. (B) Growth curves of control and RNAi root lines. Each data point represents the mean value with the standard error obtained from three independent experiments. The quality of the initial inoculation for all lines was 0.5 ± 0.001 g.

Fig. 7. The expression analysis of different upstream genes in control and RNAi root lines by quantitative real-time PCR. (a) qPCR analysis of dammarenediol synthase (*Pq-DS*), protopanaxadiol synthase (*Pq-D12H*), protopanaxatriol synthase (*Pq-P6H*) and UDP-glycosyltransferase (*Pq3-O-UGT2*) in control and RNAi root lines of *P. quinquefolius*. Actin gene was used for loading control. (b) qPCR analysis of dammarenediol synthase (*Pg-DS*), protopanaxadiol synthase (*Pg-CYP716A47*), protopanaxatriol synthase (*Pg-CYP716A53V2*) and UDP-glycosyltransferase (*Pg-UGT94Q2*) in control and RNAi root lines of *P. quinquefolius*. Actin gene was used for loading control. (c) Densitometric analysis of *Pq-DS*, *Pq-D12H*, *Pq-P6H*, and *Pq3-O-UGT2* in *P. quinquefolius* transgenic lines. (d) Densitometric analysis of *Pg-DS*, *Pg-CYP716A47*, *Pg-CYP716A53V2*, and *Pg-UGT94Q2* in *P. ginseng* transgenic lines. Each *asterisk* above the *error bar* indicates significant differences between transgenic and control samples, determined using Student *t* test (* $p \leq 0.05$).

Table 1 Ginsenoside contents in the extracts from control and RNAi root lines

Table 2 Primers designed for qPCR analysis

2

3 **Fig. 8** The proposed biosynthesis pathway of ginsenosides in *P. quinquefolius*. MVA
 4 mevalonic acid, MEP methylerythritol 4-phosphate, IPP isopentenyl diphosphate, β -
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Fig. 9 Phylogenetic tree constructed based on the deduced amino acid sequences of *Pq3-O-UGT2* and other glycosyltransferase genes of plant. *DcsUGT* (XP_017250139.1), *EuGT* (AHX74090.1), *FvsUGT* (XP_004303956.1), *NtUGT* (NP_001313072.1), *NtUGT6* (XP_009587669.1), *CcGT* (CDP16611.1), *NsUGT6-like* (XP_009799026.1), *StUGT6-like* (XP_006353724.1), *PpGT* (XP_007216098.1), *CrGT* (BAF75901.1), *VvGT6-like* (XP_010648215.1), *EgGT* (KCW88565.1), *EgGT2-like* (XP_010045071.1), *SlUGT6-like* (XP_004243651.1), *SpUGT6-like* (XP_015082329.1), *EgGT2-like* (XP_010045032.1), *CrUGT* (BAD29721.1), *PgUGT1* (AIE12479.1), *Pq20-O-UGT1* (AKI06370.1), *PgUGT29* (AKA44579.1), *PgUGT94B1* (AGR44632.1), *DcsGT-like* (XP_017249390.1), *BcGT* (AGP25113.1), *StGT-like* (XP_006361439.1), *NtGT-like* (XP_009604940.1), *NtGT-like2* (XP_016476450.1), *SpGT-like* (XP_015057251.1), *SlGT-like* (XP_004249996.1), *CaGT-like* (XP_016549951.1), *Vvg-GT* (XP_002264771.2), *NsGT-like* (XP_009770958.1), *VvGT-like* (XP_002271587.2), *BcUGT5* (AFK79037.1),

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3
4 **Fig. 10** Total ion chromatogram of HPLC/ESIMS analysis of the products of *in vitro*
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9 reaction fed with ginsenoside F2. (e) LC chromatogram of the extracts from the
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11

12

Fig. 11 HPLC/ESIMS spectra of peak A, B, C and D detected in the extracts of *in vitro* enzymatic reaction. Upper panel shows the MS spectrum of the authentic ginsenoside Rg3 (peak A) detected at a retention time of 41.87 min and Rd standard (peak B) detected at a retention time of 31.42 min, respectively. Lower panel shows the MS spectrum of the products of enzymatic reaction (peak C and D) detected in the at a retention time of 41.87 min and 31.42 min, respectively. The mass spectrometry spectrum of the products of Rg3 and Rd were the same as that of the standards.

Fig. 12 HPLC analysis of ginsenosides contents in control and RNAi root lines. Two control lines and four RNAi lines were selected from *P. ginseng* and *P. quinquefolius*, respectively. Each column represents the mean value with the standard error obtained

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Table 3 Ginsenoside contents in the extracts from control and RNAi root lines

Line	Ginsenoside (mg/g DW) ^a							Total
	Rb1	Rb2	Rc	Rd	Rg3	Rg1	Re	
C11 ^b	1.62±0.1	0.92±0.0	0.32±0.0	2.51±0.0	0.04±0.0	3.12±0.2	1.13±0.2	9.66±0.81
	5	8	7	4	1	4	2	
C12	1.57±0.1	0.94±0.0	0.41±0.1	2.48±0.1	0.03±0.0	3.21±0.1	1.07±0.2	9.71±0.93
	4	6	1	6	1	7	8	
R13	0.78±0.1	0.67±0.0	0.11±0.0	1.25±0.0	--	3.79±0.1	1.15±0.1	7.64±0.52
	1	6	9	8	--	3	4	
R14	0.69±0.0	0.72±0.0	0.12±0.0	1.47±0.0	--	3.82±0.0	1.18±0.1	7.88±0.51
	6	9	8	7	--	9	9	
R15	0.52±0.0	0.59±0.0	0.09±0.0	1.13±0.1	--	3.32±0.1	1.31±0.2	6.84±0.57
	3	8	9	2	--	1	3	
R16	0.61±0.0	0.73±0.1	0.10±0.0	1.21±0.0	--	3.67±0.0	1.26±0.1	7.48±0.44
	5	1	6	9	--	8	1	
C21	2.87±0.2	1.94±0.1	0.98±0.1	3.67±0.1	0.05±0.0	1.86±0.0	1.02±0.0	12.39±0.8
	1	7	2	7	1	7	9	
C22	2.93±0.1	2.02±0.1	0.86±0.0	3.59±0.1	0.06±0.0	1.82±0.0	1.13±0.0	12.41±0.6
	8	3	9	1	1	5	7	
R23	1.52±0.0	1.05±0.0	0.33±0.0	1.31±0.0	--	2.01±0.1	1.21±0.0	7.43±0.44
	9	8	1	4	--	9	3	
R24	1.47±0.0	1.12±0.0	0.41±0.0	1.42±0.0	--	1.97±0.1	1.17±0.0	7.56±0.35
	6	4	2	3	--	1	9	
R25	1.13±0.0	1.07±0.0	0.36±0.0	1.57±0.0	--	1.86±0.0	1.08±0.1	7.07±0.31
	2	6	1	3	--	8	1	
R26	1.34±0.0	1.08±0.0	0.38±0.0	1.52±0.0	--	1.92±0.0	1.11±0.0	7.35±0.33
	5	7	3	5	--	7	6	

Rg1, Re, Rf, Rb1, Rb2, Rc, and Rd indicates ginsenoside Rg1, -Re, -Rf, -Rb1, -Rb2, and -Rc, respectively.

^a Data represent the mean value ±standard error of three independent experiments.

^b C11 and C12 represent two control lines from *P. ginseng*; R13, R14, R15 and R16 represent four RNAi lines from *P. ginseng*, whereas C21 and C22 represent two control lines from *P. quinquefolius*; R23, R24, R25 and R26 represent four RNAi lines from *P. quinquefolius*.

1 **Table 4** Primers designed for qPCR analysis

Genes	Accession no.		Primers
Dammarenediol synthase	<i>Pg-DS</i>	AB26517 0	F: 5'-CAGTGAAGCAGTTCGTAAGGGTGTT-3'
	<i>Pq-DS</i>	KC31604 8	R: 5'-CATAGCCCATGATGTTTGTACTAGATT GT-3'
protopanaxadiol synthase	<i>Pg- CYP716A47</i>	JN604537	F: 5'-TAGTCCAAGTTTGGTTCCCGAGCAG-3'
	<i>Pq- D12H</i>	JX569336	R: 5'-CGGTCTTAATGGCACGATTCATAGC-3'
Protopanaxatriol synthase	<i>Pg- CYP716A53V2</i>	JX036031	F: 5'-GGAACGGGAGTACAGAAGTGAAAGC-3 ,
	<i>Pq- CYP6H</i>	KC19049 1	R: 5'-CTGCAAGAAAGTAGACCACAGAGCC-3'
Glycosyltransfera se	<i>Pg-UGT94Q2</i>	JX898530	F: 5'-TTGGTCCACTCGTTCAAGATCCTAT-3'
	<i>Pq-3-O-UGT2</i>	KR10620 7	R: 5'-AACACCACTGTAGATTCAGCCCTTT-3'

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