Alteration of *Panax ginseng* saponin composition by overexpression and RNA interference of the protopanaxadiol 6-hydroxylase gene (CYP716A53v2)

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**Abstract**

**Background:** The roots of *Panax ginseng* contain noble tetracyclic triterpenoid saponins derived from dammarenediol-II. Dammarene-type ginsenosides are classified into the protopanaxadiol (PPD) and protopanaxatriol (PPT) groups based on their triterpene aglycone structures. Two cytochrome P450 (CYP) genes (CYP716A47 and CYP716A53v2) are critical for the production of PPD and PPT aglycones, respectively. CYP716A53v2 is a protopanaxadiol 6-hydroxylase that catalyzes PPT production from PPD in *P. ginseng*.

**Methods:** We constructed transgenic *P. ginseng* lines overexpressing or silencing (via RNA interference) the CYP716A53v2 gene and analyzed changes in their ginsenoside profiles.

**Result:** Overexpression of CYP716A53v2 led to increased accumulation of CYP716A53v2 mRNA in all transgenic roots compared to nontransgenic roots. Conversely, silencing of CYP716A53v2 mRNA in RNAi transgenic roots resulted in reduced CYP716A53v2 transcription. HPLC analysis revealed that transgenic roots overexpressing CYP716A53v2 contained higher levels of PPT-group ginsenosides (Rg1, Re, and Rf) but lower levels of PPD-group ginsenosides (Rb1, Rc, Rb2, and Rd). By contrast, RNAi transgenic roots contained lower levels of PPT-group compounds and higher levels of PPD-group compounds.

**Conclusion:** The production of PPD- and PPT-group ginsenosides can be altered by changing the expression of CYP716A53v2 in transgenic *P. ginseng*. The biological activities of PPD-group ginsenosides are known to differ from those of the PPT group. Thus, increasing or decreasing the levels of PPT-group ginsenosides in transgenic *P. ginseng* may yield new medicinal uses for transgenic *P. ginseng*.

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1. Introduction

Triterpenoid saponins are present in many higher plants and exhibit a wide range of biological activities depending on their structures. Certain triterpenoid saponins also have commercial value and are exploited as drugs and medicines [1–3]; and saponins may have natural roles in the defense against pathogens and pests [4].

The roots of ginseng (*Panax ginseng* Meyer) contain pharmacologically active components. It is generally believed that ginsenoside saponins are the main compounds responsible for the pharmacological activities of *P. ginseng* [2,3]. Ginsenosides are classified as protopanaxadiol (PPD) or protopanaxatriol (PPT) saponins based on their aglycone structures. PPD-group ginsenosides have glycosidic bonds at the C-3 and C-20 hydroxyl groups, and the major ginsenosides of this group include Ra1, Ra2, Rb1, Rc, Rd, and Rg3. PPT-group ginsenosides have glycosidic bonds at the C-6 and C-20 hydroxyl groups, and the major ginsenosides of this group include Re, Rf, Rg1, Rg2, and Rh1. The first step in ginsenoside biosynthesis is the cyclization of 2,3-oxidosqualene to dammarenediol-II, which is catalyzed by dammarenediol synthase of the oxidosqualene cyclase group [5].

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Dammarenediol-II is hydroxylated by two cytochrome P450 enzymes (CYP716A47 and CYP71653v2) to produce PPDs and PPTs [6,7]. These compounds are subsequently glycosylated by glycosyltransferase (Fig. 1). The CYP716A47 enzyme in *P. ginseng* catalyzes the hydroxylation of dammarenediol-II at the C-12 position to yield PPD, which is further hydroxylated at its C-6 position by CYP71653v2 to yield PPT.

PPD-group ginsenosides have biological activities that are opposite of those in the PPT group [8,9]. For example, Rg1 (PPD group) suppresses the activity of the central nervous system [8,9], whereas Rb1 (PPD group) stimulates the central nervous system, whereas Rb1 (PPD group) suppresses the activity of the central nervous system [8,9]. For example, Rg1 (PPD group) suppresses the activity of the central nervous system [8,9].

Because the two CYP genes (CYP716A47 and CYP71653v2) are necessary for producing PPD and PPT aglycones, respectively, we postulated that modifying the expression of these genes in *P. ginseng* could alter the composition of ginsenosides. Transgenic *P. ginseng* with altered ginsenoside profiles could be used for new medicinal applications requiring precise tuning of pharmacological activity.

In this study, we constructed two transgenic *P. ginseng* lines, with overexpression or silencing [RNA interference (RNAi)] of CYP716A53v2, and we analyzed the changes in their PPD and PPT ginsenoside levels. We found that the production of PPT-group ginsenosides was altered by both the overexpression and RNAi-based silencing of the CYP716A53v2 gene.

2. Materials and methods

2.1. Overexpression and RNAi silencing vector construction

The ORF (open reading frame) region of the CYP716A53v2 sequence was cloned into the pCR 8.0 vector (Invitrogen Life Technologies, Carlsbad, CA, USA) and then transferred to the destination vector pH2WG to yield the CYP716A53v2 overexpression vector. To construct the CYP716A53v2-RNAi vector, two primers including gateway adapters (Invitrogen Life Technologies) were designed to amplify the region from 1,259 bp to 1,498 bp in CYP716A53v2. The amplified polymerase chain reaction (PCR) product was cloned into the pSB1 vector and then transferred to the RNAi destination vector pB7GW1WG2(II) (which contains the BAR gene that confers Basta resistance to plant cells) in *Escherichia coli* DH5α, as described by the manufacturer (Invitrogen Life Technologies). The construct was sequenced and subsequently transformed into *Agrobacterium tumefaciens* GV3101 cells harboring plasmid pMP90 using standard molecular biology techniques.

2.2. Construction of transgenic *P. ginseng*

The generation of transgenic *P. ginseng* was conducted as described in our previous report [10]. Putative transgenic somatic embryos for both CYP716A53v2 overexpression and CYP716A53v2-RNAI were transferred to the selection medium, with additional supplementation of 20μM GA3, to induce embryo germination. The plantlets were maintained on 1/2-strength MS medium with 2% sucrose.

Selection of transgenic root lines was performed as described by Han et al [5]. As a nontransgenic control, adventitious roots were induced from the in vitro maintained nontransformed plants that were the original sources of the transgenic plants.

2.3. Reverse transcription-PCR in transgenic roots

Total RNA was isolated from nontransgenic and transgenic roots and reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). First-strand cDNA was used as the template for the reverse transcription (RT)-PCR analysis, which was performed as follows: 96°C for 5 min; 30 cycles of 96°C for 30 s, 60°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 10 min. β-Actin cDNA (primers 5'-ATG GTC AAG GCT GGA TTT GCA-3' and 5'-CTC GAC CAG CTA AAT CAA GAC-3') was used as a control for RNA integrity and loading accuracy. The RT-PCR analyses were performed twice, and representative data are shown in the figures. The primers used for amplification were 5'-ATG CAT CCT TTC ATT TCA TCT CAA-3' and 5'-CTA AAG CGT ACA AGG TGA TAG AGC-3' for *P. ginseng* CYP716A53v2, 5'-GGC TGA CCT ATT GCA TCT CCA-3' and 5'-TTC TAC ACA GCC ATC GGT CTC CC-3' for the hygromycin phosphotransferase gene (HPT), and 5'-AGG ACA GAG CCA ACA ACA CC-3' and 5'-ATG CTT GGA TCC TCC G-3' for the phosphinothricin acetyl transferase gene (BAR).

**Fig. 1.** Biosynthetic pathway for ginsenosides in *Panax ginseng*. Squalene epoxidase converts squalene to 2,3-oxidosqualene, which is then converted to a triterpene aglycone (dammarenediol-II) by dammarenediol synthase. Dammarenediol-II undergoes oxidation and glycosylation and is finally converted to protopanaxadiol (PPD)- and protopanaxatriol (PPT)-group ginsenosides.
2.4. Quantitative PCR in transgenic roots

Quantitative PCR (qPCR) was performed using a Qiagen Rotor Gene Q real-time PCR detector system (Qiagen, Hilden, Germany) with SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). The cycling parameters for the qRT-PCR were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 5 s and 60°C for 10 s. The qRT-PCR data shown are the average relative quantities ± standard error from at least three replicates. The relative expression level of each gene was calculated using the ΔΔCt method [11]. The P. ginseng β-actin gene was used for normalization. The primers used for the amplification were as follows: 5′-GGC CCC CTG CCA TAT CCA AAA TCC-3′ and 5′-CAA AGT GCT GCC TCG GGA TTT GCA-3′ for P. ginseng CYP716A53v2; 5′-ATG GTC AAG GCT GGA TTT GCA-3′ and 5′-GAG CCT CAT ATC CAA CAT ATG C-3′ for P. ginseng β-actin; 5′-ATG CGG AAA AGC AGA TCC TTT GCA-3′ and 5′-AAA TGG TGG TAT CAA GAG GAT TCT GAT TCT CTT C-3′ for PgSS1; 5′-AAG CTA GCG TTC TCG TCC TT-3′ and 5′-TTG CTA CGT CGG TAC TTC CG-3′ for PgSQE1; 5′-ATA TAT CAG CGT CTA ATG CAC-3′ and 5′-ATT CAG CTA ATC ATC GAT TCG AGA AAA TTT GCA-3′ for PgPNX.

2.5. Ginsenoside analysis by HPLC

Extraction and analysis of ginsenosides was performed using the method described by Han et al [6]. The HPLC separation was performed using a Cosmosil C18 column (5 μm, 4.6 × 250 mm; Agilent, Santa Rosa, CA, USA). The time course measurements and measurements of the ratios of water and acetonitrile were performed according to the protocol reported by Han et al [6]. Each ginsenoside was compared to authentic ginsenoside samples purchased from ChromaDex, Inc. (Irvine, CA, USA).

3. Results

3.1. Overexpression and RNA interference of CYP716A53v2 in transgenic P. ginseng

Transgenic ginseng plants overexpressing CYP716A53v2 were generated using A. tumefaciens-mediated genetic transformation (Fig. 2A). Transgenic root lines were induced and selected according to the protocol by Han et al [5]. Seven transgenic overexpression lines were finally selected for further analysis. The RT-PCR analysis revealed clear transcription of the selection marker, hygromycin phosphotransferase (HPT), in the overexpression lines (Fig. 2B). Each ginsenoside was compared to authentic ginsenoside samples purchased from ChromaDex, Inc. (Irvine, CA, USA).

Fig. 2. Expression of introduced genes in transgenic ginseng roots overexpressing CYP716A53v2. (A) T-DNA region of the plasmid for CYP716A53v2 gene overexpression under the control of the CaMV35S promoter. T35S, CaMV 35S terminator; HPT, hygromycin phosphotransferase gene; RB, right border of the T-DNA; LB, left border of the T-DNA. (B) Confirmation of transcription of the introduced genes (HPT and CYP716A53v2) by reverse transcription–polymerase chain reaction (RT-PCR). β-Actin was used as a loading control. (C) Transcription of CYP716A53v2 in nontransgenic (Nt) and transgenic lines (1–7) overexpressing CYP716A53v2, based on quantitative PCR (qPCR). Data are shown as mean values with the standard error obtained from three independent plants. Asterisks indicate significant differences between nontransgenic and transgenic plants (Student’s t test, p ≤ 0.05).
lines were clearly reduced compared to nontransgenic roots (Fig. 3C). As measured by qPCR, each of the CYP716A53v2-RNAi lines showed a decrease of at least 75% in the level of the CYP716A53v2 transcript.

3.2. Altered ginsenoside profiles in transgenic P. ginseng

The HPLC chromatogram profiles revealed that PPT-group ginsenosides (Rg1, Re, and Rf) were clearly reduced in the transgenic RNAi line (line RNAi 3) (Fig. 4B) and were increased in the overexpressing transgenic line (line over 7; Fig. 4C) compared with those in the control (Fig. 4A). By contrast, PPD-group ginsenosides (Rb1, Rb2, Rc, and Rd) were clearly enhanced in transgenic RNAi roots (Fig. 4B) but reduced in overexpressing transgenic roots (Fig. 4C).

The levels of PPT-group ginsenosides (Rg1, Re, and Rf) were increased by at least 1.5-fold in all seven of the overexpression lines relative to nontransgenic control roots (Table 1, Fig. 5A). However, the levels of PPD-group ginsenosides (Rc, Rd, Rb1, and Rb2) were reduced in overexpression lines compared with the controls. The total ginsenoside levels in roots of the overexpression lines were increased compared to those in the nontransgenic controls (Fig. 5A). In transgenic RNAi lines, the levels of PPT-group ginsenosides (Rg1, Re, and Rf) were clearly reduced compared with those in nontransgenic roots (Table 2, Fig. 5B). In line 3, the PPT-group ginsenosides were nearly absent. Interestingly, the total amount of PPD-group ginsenosides (Rc, Rd, Rb1, and Rb2) was markedly increased in all RNAi lines compared with the nontransgenic roots (Fig. 5B). The total ginsenoside content was lowered in transgenic RNAi lines compared with nontransgenic roots (Fig. 5B and Table 2).

3.3. Expression of genes involved in ginsenoside biosynthesis in transgenic lines

Squalene synthase is the first enzymatic step leading from the central isoprenoid pathway to ginsenoside biosynthesis [12]. It has been reported that PgSS1 in P. ginseng is a regulatory enzyme not only for phytosterol but also for triterpene biosynthesis [12]. Squalene epoxidase catalyzes the first oxygenation step in ginsenoside biosynthesis and is thought to be one of the rate-limiting enzymes in this pathway. Han et al [13] reported that PgSQE1 and PgSQE2 are regulated via different mechanisms and that PgSQE1 regulates ginsenoside biosynthesis but not phytosterol biosynthesis. The production of the ginsenoside triterpene skeleton relies on the cyclization of 2,3-oxidosqualene to dammarenediol-II catalyzed by dammarenediol synthase (PgDDS) [5]. Han et al [6] reported that CYP716A47 is responsible for the hydroxylation of dammarenediol-II at the C-12 position to yield PD. Cycloartenol synthase (PgPNX) in ginseng is involved in the production of cycloartenol, which is the precursor for phytosterol biosynthesis.

The transcription levels of genes related to ginsenoside biosynthesis, such as PgSS1, PgSQE1, PgDDS, CYP716A47, and PgPNX, were measured with qPCR in selected lines of transgenic ginseng (overexpression and RNAi; Figs. 6, 7). Overexpression and RNA interference of CYP716A53v2 did not affect the expression of upstream genes in the pathway (PgSS1, PgSQE1, PgDDS, and CYP716A47); there was no clear change in mRNA accumulation between the nontransgenic control and the transgenic roots (Figs. 6, 7). Moreover, there was no difference in the accumulation of PgPNX mRNA between transgenic roots and nontransgenic roots (Figs. 6, 7).
4. Discussion

4.1. Altered ginsenoside composition in transgenic P. ginseng

Transgenic ginseng plants were constructed to either over-express or silence the CYP716A53v2 gene. RT-PCR and qPCR analyses revealed that all of the transgenic ginseng roots overexpressing CYP716A53v2 showed increased CYP716A53v2 transcription. All of the CYP716A53v2-RNAi roots showed reduced accumulation of CYP716A53v2 mRNA.

Ginsenoside analysis by HPLC revealed that the transgenic ginseng roots overexpressing CYP716A53v2 contained higher levels of PPT-group ginsenosides (Rg1, Re, and Rf), whereas the CYP716A53v2-RNAi roots contained lower levels. These results indicate that the production of PPT-group ginsenosides is affected by the engineered expression of CYP716A53v2. Overproduction of PPTs in CYP716A53v2-overexpressing roots reduced the levels of PPDs. Conversely, reduced PPT production in the CYP716A53v2-RNAi roots stimulated the production of PPDs. These results indicate that PPD and PPT production may be competing pathways that...
Ginsenoside concentration in RNAi transgenic lines silencing the CYP716A53v2 gene

| Line   | PPT type | Ginkgoside concentration (mg/g dry weight) | PPD type | Total |
|--------|----------|--------------------------------------------|----------|-------|
|        | Rg1      | Re                                         | Rf       | Rb1   | Rc    | Rb2   | Rd    |
| Nt     | 2.73 ± 0.28 | 1.03 ± 0.18 | 0.11 ± 0.13 | 1.56 ± 0.19 | 0.19 ± 0.17 | 0.51 ± 0.02 | 0.17 ± 0.11 | 6.30 ± 0.62 |
| RNAI 1 | 0.45 ± 0.03 | 0.09 ± 0.01 | —          | 2.62 ± 0.12 | 0.32 ± 0.02 | 0.55 ± 0.03 | 0.27 ± 0.03 | 4.30 ± 0.2 |
| RNAI 2 | 0.52 ± 0.06 | 0.08 ± 0.01 | —          | 2.43 ± 0.17 | 0.26 ± 0.01 | 0.46 ± 0.02 | 0.26 ± 0.03 | 4.01 ± 0.17 |
| RNAI 3 | 0.07 ± 0.01 | —          | —          | 1.88 ± 0.21 | 0.34 ± 0.02 | 0.48 ± 0.04 | 0.28 ± 0.02 | 3.05 ± 0.18 |
| RNAI 4 | 0.28 ± 0.02 | —          | —          | 2.26 ± 0.19 | 0.44 ± 0.03 | 1.2 ± 0.13 | 0.23 ± 0.02 | 4.41 ± 0.27 |
| RNAI 5 | 0.35 ± 0.03 | 0.08 ± 0.01 | —          | 2.67 ± 0.31 | 0.53 ± 0.04 | 0.86 ± 0.07 | 0.24 ± 0.01 | 4.73 ± 0.27 |
| RNAI 6 | 1.29 ± 0.13 | 0.2 ± 0.03 | —          | 1.83 ± 0.19 | 0.35 ± 0.02 | 0.56 ± 0.04 | 0.24 ± 0.03 | 4.47 ± 0.25 |
| RNAI 7 | 1.06 ± 0.11 | 0.1 ± 0.01 | —          | 2.53 ± 0.22 | 0.26 ± 0.01 | 0.39 ± 0.04 | 0.26 ± 0.03 | 4.60 ± 0.24 |

Table 2
Ginsenoside concentration in RNAI transgenic lines silencing the CYP716A53v2 gene

| Line   | PPT type | Girkoside concentration (mg/g) | PPD type | Total |
|--------|----------|--------------------------------|----------|-------|
|        | Rg1      | Re                                         | Rf       | Rb1   | Rc    | Rb2   | Rd    |
| Nt     | 2.73 ± 0.28 | 1.03 ± 0.18 | 0.11 ± 0.03 | 1.56 ± 0.19 | 0.19 ± 0.17 | 0.51 ± 0.02 | 0.17 ± 0.11 | 6.30 ± 0.62 |
| RNAI 1 | 0.45 ± 0.03 | 0.09 ± 0.01 | —          | 2.62 ± 0.12 | 0.32 ± 0.02 | 0.55 ± 0.03 | 0.27 ± 0.03 | 4.30 ± 0.2 |
| RNAI 2 | 0.52 ± 0.06 | 0.08 ± 0.01 | —          | 2.43 ± 0.17 | 0.26 ± 0.01 | 0.46 ± 0.02 | 0.26 ± 0.03 | 4.01 ± 0.17 |
| RNAI 3 | 0.07 ± 0.01 | —          | —          | 1.88 ± 0.21 | 0.34 ± 0.02 | 0.48 ± 0.04 | 0.28 ± 0.02 | 3.05 ± 0.18 |
| RNAI 4 | 0.28 ± 0.02 | —          | —          | 2.26 ± 0.19 | 0.44 ± 0.03 | 1.2 ± 0.13 | 0.23 ± 0.02 | 4.41 ± 0.27 |
| RNAI 5 | 0.35 ± 0.03 | 0.08 ± 0.01 | —          | 2.67 ± 0.31 | 0.53 ± 0.04 | 0.86 ± 0.07 | 0.24 ± 0.01 | 4.73 ± 0.27 |
| RNAI 6 | 1.29 ± 0.13 | 0.2 ± 0.03 | —          | 1.83 ± 0.19 | 0.35 ± 0.02 | 0.56 ± 0.04 | 0.24 ± 0.03 | 4.47 ± 0.25 |
| RNAI 7 | 1.06 ± 0.11 | 0.1 ± 0.01 | —          | 2.53 ± 0.22 | 0.26 ± 0.01 | 0.39 ± 0.04 | 0.26 ± 0.03 | 4.60 ± 0.24 |

Nt, nontransgenic; PPD, protopanaxadiol; PPT, protopanaxatriol; RNAI, RNA interference
Fig. 6. Quantitative polymerase chain reaction (qPCR) for genes upstream of ginsenoside biosynthesis in CYP716A53v2-overexpressing transgenic roots of Panax ginseng. Transcription of PgSS1, PgSQE1, PgDDS, CYP716A47, and PgPNX in transgenic lines.

Fig. 7. Quantitative polymerase chain reaction (qPCR) for genes upstream of ginsenoside biosynthesis in CYP716A53v2-RNAi transgenic roots of Panax ginseng. Transcription of PgSS1, PgSQE1, PgDDS, CYP716A47, and PgPNX in transgenic lines.
phytosterols (β-sitosterol, stigmasterol, and campesterol) and ginsenosides [12]. However, all of the above reports address the control of total ginsenoside biosynthesis. Our report is the first to describe fine-tuning the biosynthesis of specific ginsenoside groups.

The medicinal effects of these ginsenosides have been extensively studied, and they are involved in the immune system, anti-stress effects, anti-inflammatory activity, anti-oxidant activity, and anticancer effects [3,18]. In general, the PPD-group ginsenosides have pharmaceutical activities that are opposite those of the PPT group [8,9]. Therefore, transgenic ginseng plants containing increased or reduced levels of PPT-group ginsenosides due to altered expression of CYP716A53v2 could be used as new medicinal plants with specific pharmacological activities.

The physiological role of saponins in plants is not yet fully understood. Saponins may play important roles in plant defense. The bitter taste of ginsenosides makes them antifeedants [19–23]. Various biological roles have been proposed for different saponins, mostly involving allelopathic activity and defense against insects and pathogens [22]. Ginsenosides have antimicrobial [24] and antifungal activities in nature [25]. Ginsenosides also have antiviral activities toward human viruses [26]. Lee et al. [27] reported that transgenic tobacco producing dammarenediol-II by overexpression of the PgDDS gene confers resistance to tobacco mosaic virus by inhibiting viral replication.

The fungus Cylindrocarpon destructans causes serious root rot disease in ginseng-producing areas and is a main cause of ginseng replant failure [28]. PPT- and PPD-group ginsenosides exhibit different activities on C. destructans: PPT-group ginsenosides inhibit its growth, whereas PPD-group ginsenosides significantly enhance its growth [25]. However, simply increasing the level of total ginsenosides did not prevent the fungal pathogen from invading the ginseng [25]. Thus, specifically increasing the levels of PPT-group ginsenosides in transgenic ginseng by overexpressing CYP716A53v2 may be necessary to provide defense against C. destructans, thereby conferring resistance to root rot disease.

In conclusion, ginsenoside levels were successfully altered by changing the expression of CYP716A53v2 in transgenic ginseng. These transgenic P. ginseng plants can be used to produce new genetically engineered cultivars with specific biological traits such as changed medicinal values and defenses against ginseng pathogens and other microorganisms.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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