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Research article

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 (Rg_1, Re, and Rf) but lower levels of PPD-group ginsenosides (Rb1, Rc, Rb_2, 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 (PPT group) stimulates the central nervous system, whereas Rb1 (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 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.

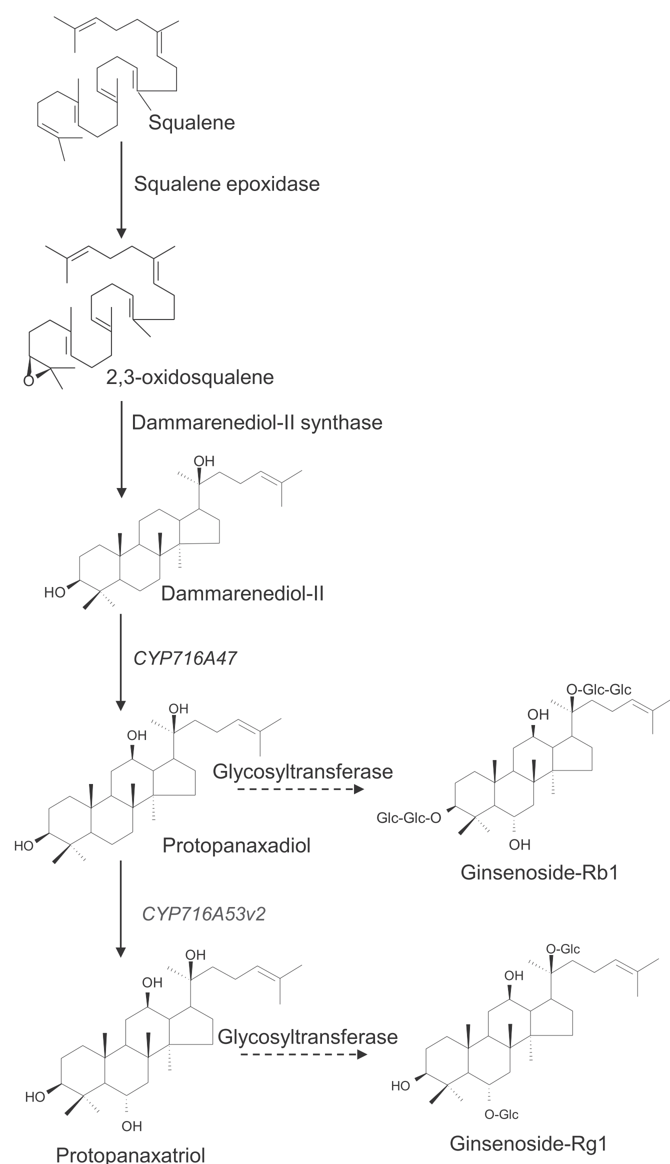

Fig. 1. Biosynthetic pathway for ginsenosides in *Panax ginseng*. Squalene epoxidase converts squalene to 2,3-oxidosqualene, which is then converted to a triterpene aglycone (dammarenediol-II) by dammarenediol synthase. Dammarenediol-II undergoes oxidation and glycosylation and is finally converted to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

2. 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 pB7GWIWG2(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 GA_3, 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 G-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 GAT CTC TTT ATC TCA TCT CAA-3′ and 5′-TTA AAG CGT ACA AGG TGA TAG ACG-3′ for *P. ginseng CYP716A53v2*, 5′-GCG TGA CCT ATT GCA TCT CC-3′ and 5′-TTC TAC ACA GCC ATC GGT CC-3′ for the hygromycin phosphotransferase gene (*HPT*), and 5′-AGG ACA GAG CCA CAA ACA CC-3′ and 5′-ATG CTT GTA TCC AGC TGC G-3′ for the phosphinothricin acetyl transferase gene (*BAR*).

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 $\Delta\Delta C_t$ 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 TTG TCC G -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 CT-3′ and 5′-AAA TGC ATA CAG CAT CGC GG-3′ for *PgSS1*; 5′-AAG CTA GCG CTC TGC TCA TT-3′ and 5′-TGA TGA CGT CCG TAC TTC CG-3′ for *PgSQE1*; 5′-ATA TAT CAG CGG AAC GAT TGA CAC-3′ and 5′-ATT CAG CTA ATC CTT CTC CTA GCA A-3′ for *PgDDS*; 5′-GCA GCA GCA ATG GTG TTG TTT-3′ and 5′-TCG CCT ATC AAA GGC CAA CC-3′ for *CYP716A47*; 5′-TTC GCA ATC ATC GAT TCG AGA AAA A-3′ and 5′-AGA CTA CAC TGC CTG GAT TCT C-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). RT-PCR showed higher transcript levels of *CYP716A53v2* in roots of all transgenic lines than in nontransgenic roots (Fig. 2C). Increases of > 2.5-fold were achieved in all seven transgenic roots, as measured by qPCR.

In transgenic ginseng plants for silencing (RNAi) *CYP716A53v2* (Fig. 3A), expression of the selection marker gene, phosphinothricin acetyl transferase (*BAR*), was clearly shown in all RNAi lines by RT-PCR analysis (Fig. 3B). *CYP716A53v2* transcript levels in all RNAi

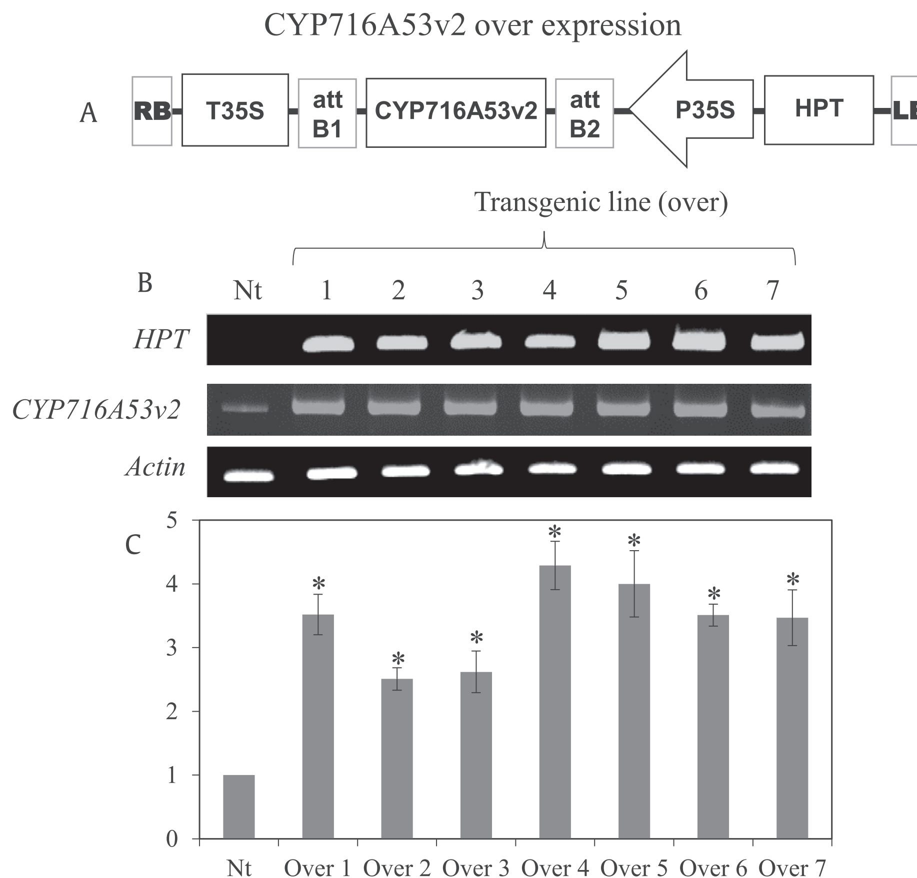

Fig. 2. Expression of introduced genes in transgenic ginseng roots overexpressing *CYP716A53v2*. (A) T-DNA region of the plasmid for *CYP71653v2* gene overexpression under the control of the CaMV35S promoter. T35S, CaMV 35S terminator; HPT, hygromycin phosphotransferase gene; RB, right border of the 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 *t* test, $p \leq 0.05$).

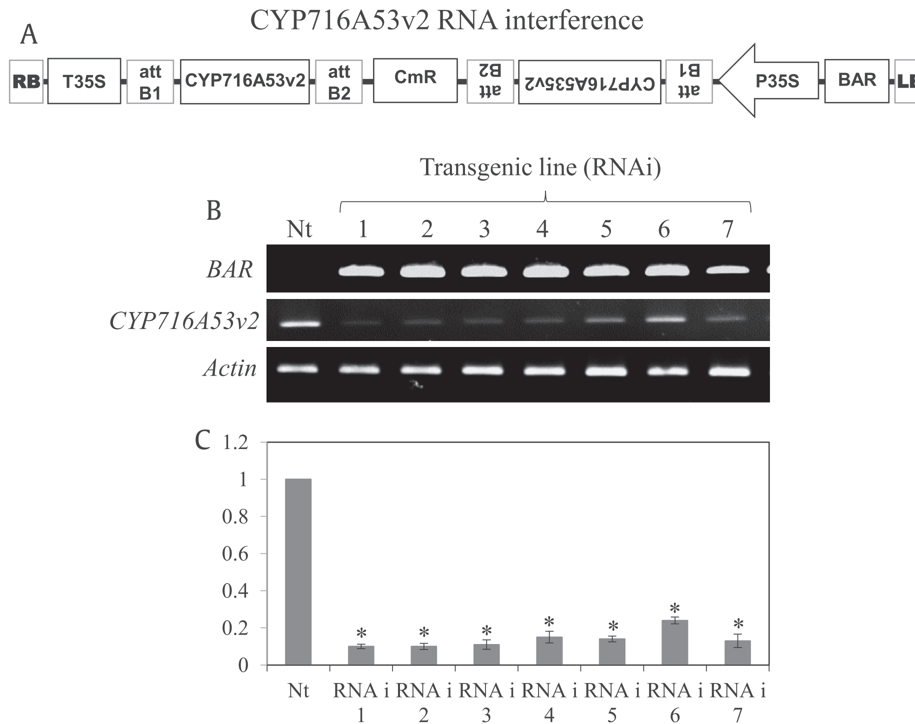

Fig. 3. Expression of introduced genes in transgenic ginseng roots overexpressing *CYP716A53v2*-RNAi. (A) The T-DNA region of the plasmid for RNAi of the CYP71653v2 gene under the control of the CaMV35S promoter. (B) Confirmation of transcription of the introduced genes (*BAR* and *CYP716A53v2*) using genomic RT-PCR. *β-Actin* was used as a loading control. (C) Transcription of *CYP716A53v2* in Nt and *CYP716A53v2*-RNAi transgenic lines 1–7, based on qPCR. Asterisks indicate significant differences between nontransgenic and transgenic plants (Student *t* test, $p \leq 0.05$). Data are shown as mean values with the standard error obtained from three independent plants. BAR, phosphinothricin acetyl transferase; Nt, nontransgenic; qPCR, quantitative PCR; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction.

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, Table 1).

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 PPD. 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).

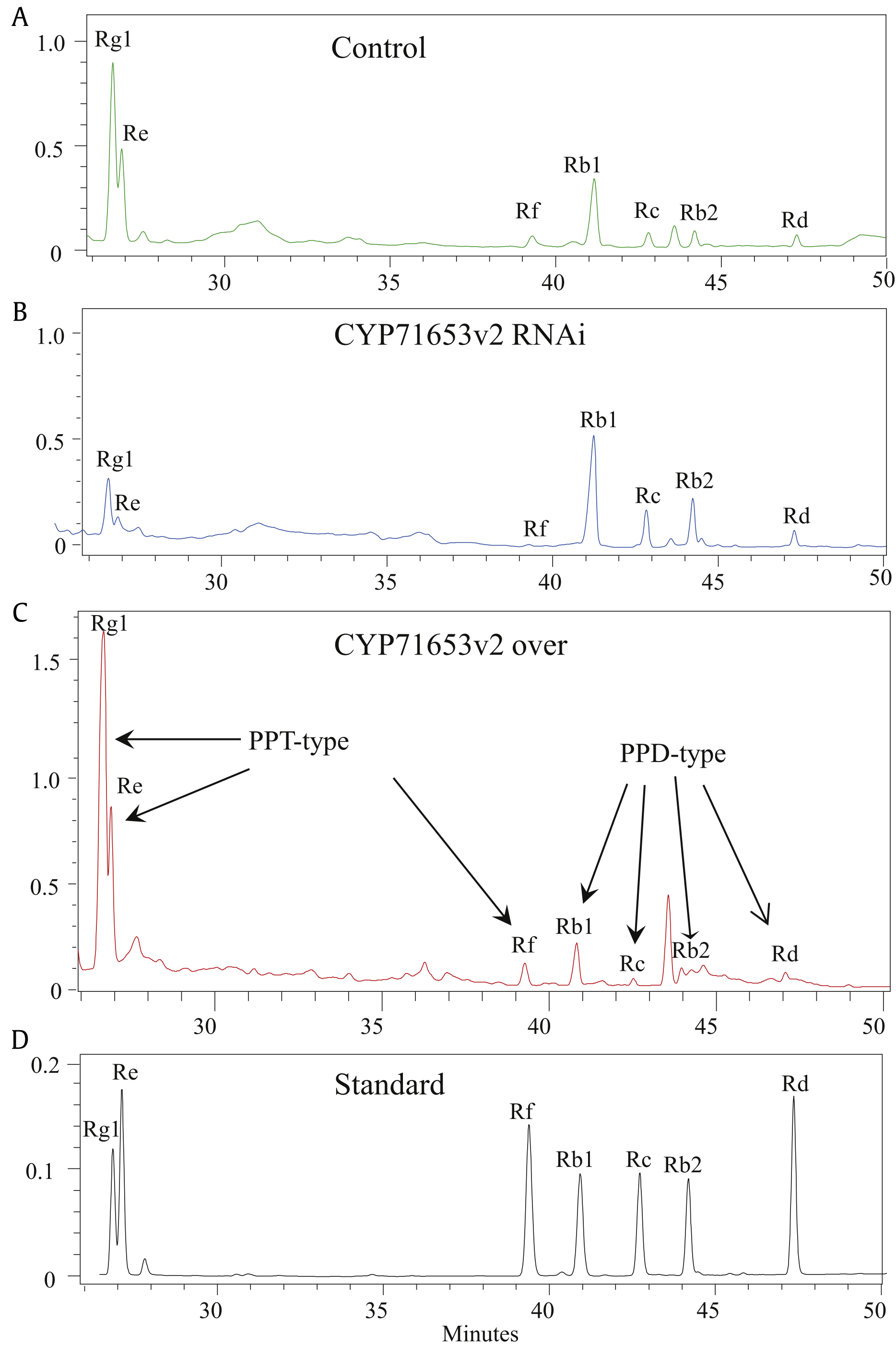

Fig. 4. Ginsenoside chromatogram of extracts from nontransgenic and transgenic roots. (A) Ginsenoside chromatograms of extracts from nontransgenic roots. (B) Transgenic *CYP716A53v2*-RNAi roots (line RNAi 3). (C) Transgenic *P. ginseng* roots overexpressing *CYP716A53v2* (line over 7). (D) HPLC chromatogram of ginsenoside standards.

4. Discussion

4.1. Altered ginsenoside composition in transgenic P. ginseng

Transgenic ginseng plants were constructed to either overexpress 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

Table 1
Ginsenoside concentrations in transgenic lines overexpressing *CYP716A53v2*

Line	Ginsenoside concentration (mg/g dry weight)							Total
	PPT type			PPD type				
	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
Over 1	6.82 ± 0.84	1.16 ± 0.12	0.22 ± 0.12	0.73 ± 0.07	0.11 ± 0.06	0.14 ± 0.01	0.12 ± 0.03	9.30 ± 0.79
Over 2	6.42 ± 0.52	1.22 ± 0.19	0.21 ± 0.22	0.64 ± 0.05	0.07 ± 0.01	0.16 ± 0.03	0.17 ± 0.02	8.89 ± 0.68
Over 3	5.55 ± 0.47	1.29 ± 0.18	0.16 ± 0.01	0.4 ± 0.05	0.1 ± 0.05	0.17 ± 0.02	0.13 ± 0.01	7.80 ± 0.5
Over 4	6.78 ± 0.61	1.35 ± 0.13	0.23 ± 0.02	0.65 ± 0.03	0.09 ± 0.01	0.16 ± 0.03	0.13 ± 0.01	9.39 ± 0.55
Over 5	4.98 ± 0.73	1.17 ± 0.15	0.17 ± 0.01	0.67 ± 0.06	0.05 ± 0.01	0.16 ± 0.02	0.18 ± 0.02	7.38 ± 0.67
Over 6	8.24 ± 0.43	1.58 ± 0.17	0.29 ± 0.03	0.56 ± 0.03	0.09 ± 0.01	0.14 ± 0.01	0.11 ± 0.01	11.01 ± 0.48
Over 7	6.17 ± 0.87	1.96 ± 0.19	0.38 ± 0.02	0.41 ± 0.03	0.03 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	9.15 ± 0.71

PPD, protopanaxadiol; PPT, protopanaxatriol

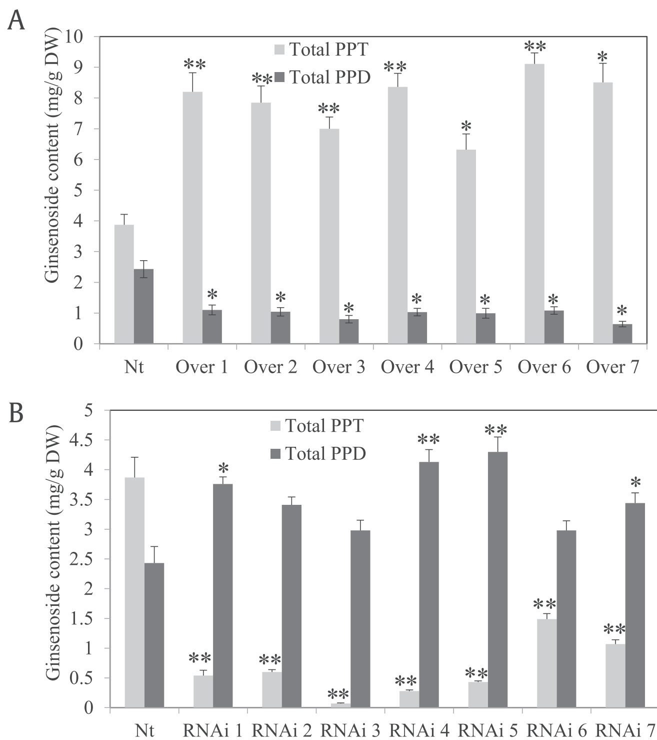

Fig. 5. HPLC analysis of ginsenosides in nontransgenic and transgenic ginseng roots. (A) Ginsenoside contents in roots of transgenic lines (over 1 to 7) overexpressing *CYP716A53v2*. (B) Ginsenoside contents in roots of *CYP716A53v2*-RNAi lines (RNAi 1 to 7). Data are shown as means ± SE ($n = 3$ each). Significance was determined using Student t test ($^{*}\, p \leq 0.05$; $^{**}\, p \leq 0.01$) for independent means. Nt, nontransgenic roots; *SE, standard error.*

both depend on a common intermediate triterpene (dammarenediol-II).

Using qPCR, we investigated the expression of upstream genes in the *CYP716A53v2* overexpression and RNAi roots. Neither overexpression nor RNAi affected the key upstream genes *PgSS1*, *PgSQE1*, *PgDDS*, and *CYP716A47*, and there was no effect on the OSC gene of the sterol pathway (*PgPNX*). These results strongly suggest that altering *CYP716A53v2* expression by using an engineered promoter does not affect the upstream genes in the ginsenoside biosynthesis pathway. The lower levels of PPD ginsenosides in *CYP716A53v2*-overexpressing roots and the increase in PPD ginsenosides in *CYP716A53v2*-RNAi roots may result from complementary use of sapogenin (PPD and PPT) precursors in *P. ginseng*. However, it is not clear why the total ginsenoside content differs between the *CYP716A53v2*-overexpressing and *CYP716A53v2*-silenced transgenic roots. To produce the final PPD and PPT ginsenosides, various types of glycosyltransferases are required. Recently, genes for a few glycosyltransferases involved in ginsenoside biosynthesis have been identified [14]. It is likely that changing the expression of *CYP716A53v2* affects the downstream genes (glycosyltransferases).

There are many reports of achieving increased ginsenoside accumulation by elicitor or precursor treatment or by overexpression of key ginsenoside biosynthesis enzymes in transgenic *P. ginseng* [15]. Furuya et al [16] reported that treatment with several intermediates and precursors involved in ginsenoside biosynthesis, e.g., mevalonate or farnesol, was effective for increasing ginsenoside production. Treatments with elicitors such as yeast extract or methyl jasmonate also enhanced the ginsenoside production [16,17]. Lee et al [12] found that squalene synthase (*PgSS1*) increased the production of both phytosterols and ginsenosides. Overexpression of the *PgSS1* gene in adventitious roots of transgenic *P. ginseng* resulted in an increase in the production of

Table 2
Ginsenoside concentration in RNAi transgenic lines silencing the *CYP716A53v2* gene

Line	Ginsenoside concentration (mg/g)							Total
	PPT type			PPD type				
	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 ± 0.51
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, nonstransgenic; PPD, protopanaxadiol; PPT, protopanaxatriol; RNAi, RNA interference

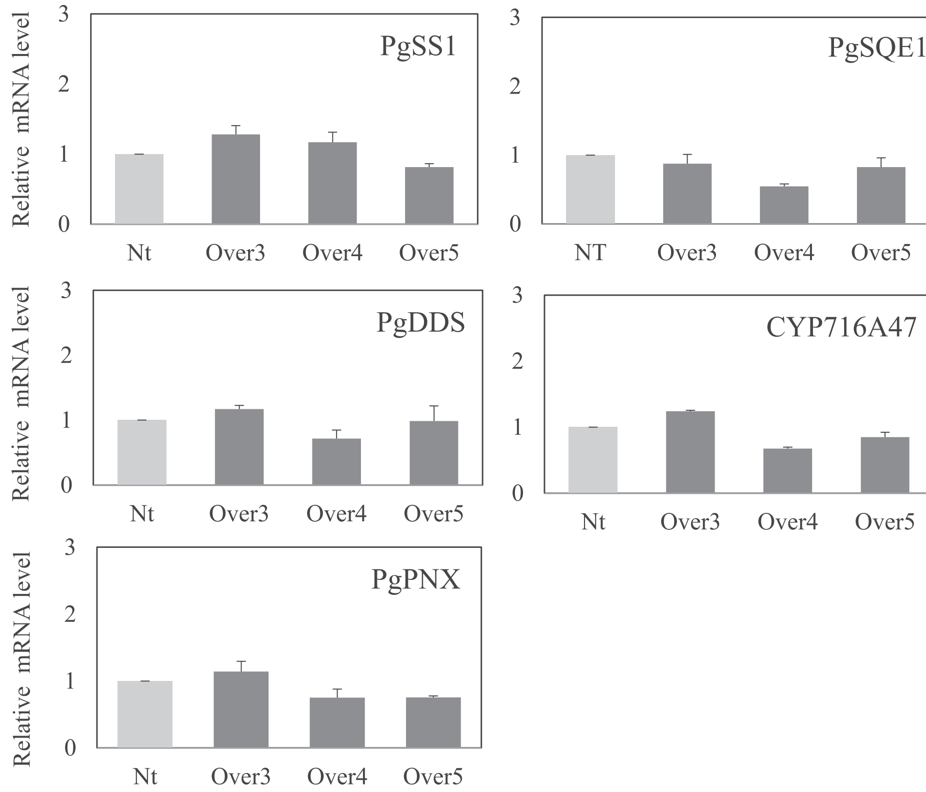

Fig. 6. Quantitative polymerase chain reaction (qPCR) for genes upstream of ginsenoside biosynthesis in *CYP716A53v2*-overexpressing transgenic roots of *Panax ginseng*. Transcription of *PgSS1*, *PgSQE1*, *PgDDS*, *CYP716A47*, and *PgPNX* in transgenic lines.

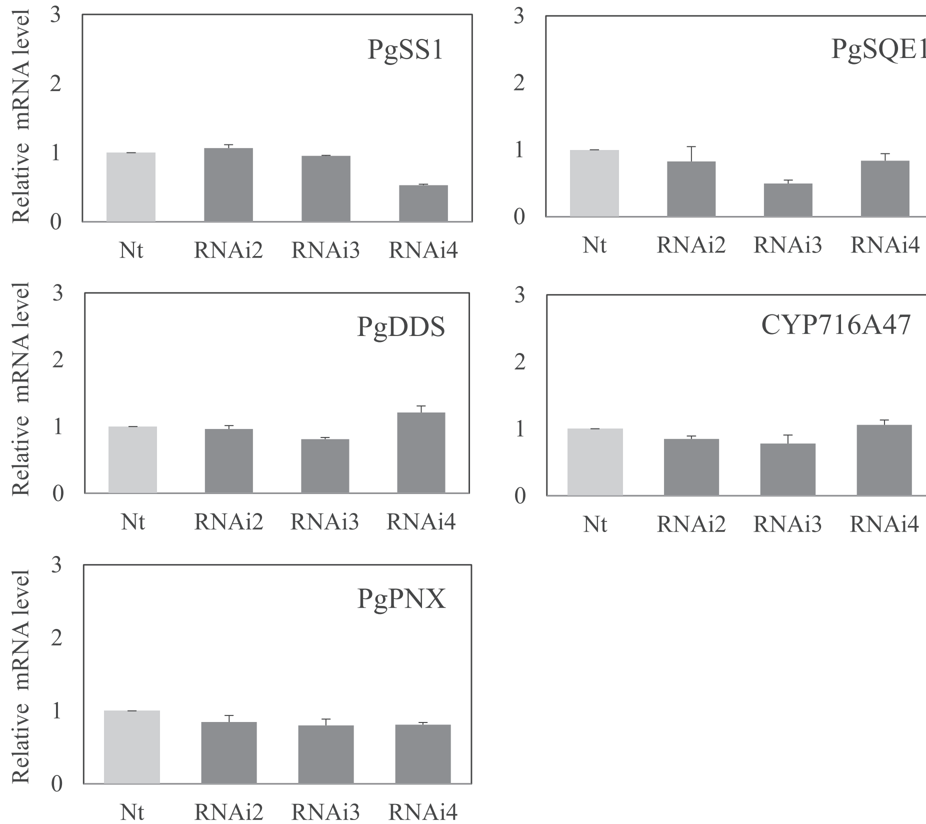

Fig. 7. Quantitative polymerase chain reaction (qPCR) for genes upstream of ginsenoside biosynthesis in *CYP716A53v2*-RNAi transgenic roots of *Panax ginseng*. Transcription of *PgSS1*, *PgSQE1*, *PgDDS*, *CYP716A47*, and *PgPNX* in transgenic lines.

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, antistress effects, antihyperglycemic activity, anti-inflammatory activity, antioxidant 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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References

[1] Hostettmann KA, Marston A. Saponins. Chemistry and pharmacology of natural products. Cambridge: United Kingdom: Cambridge University Press; 1995.
[2] Vogler BK, Pittler MH, Ernst E. The efficacy of ginseng. A systematic review of randomised clinical trials. Eur J Clin Pharmacol 1999;55:567–75.
[3] Shibata S. Chemistry and cancer preventing activities of ginseng saponins and some related triterpenoid compounds. J Korean Med Sci 2001;16:S28–37.
[4] Osbourn AE. Preformed antimicrobial compounds and plant defense againts fungal attack. Plant Cell 1996;8:1821–31.
[5] Han JY, Kwon YS, Yang DC, Jung YR, Choi YE. Expression and RNA interference-induced silencing of the dammarenediol synthase gene in *Panax ginseng*. Plant Cell Physiol 2006;47:1653–62.
[6] Han JY, Kim HJ, Kwon YS, Choi YE. The Cyt P450 enzyme CYP716A47 catalyzes the formation of protopanaxadiol from dammarenediol-II during ginsenoside biosynthesis in *Panax ginseng*. Plant Cell Physiol 2011;52:2062–73.
[7] Han JY, Hwang HS, Choi SW, Kim HJ, Choi YE. Cytochrome P450 *CYP716A53v2* catalyzes the formation of protopanaxatriol from protopanaxadiol during ginsenoside biosynthesis in *Panax ginseng*. Plant Cell Physiol 2012;53:1535–45.
[8] Sticher O. Getting to the root of ginseng. Chemtech 1998;28:26–32.
[9] Kim JH, Kang SA, Han SM, Shim I. Comparison 1 of the antiobesity effects of the protopanaxadiol- and protopanaxatriol-type saponins of red ginseng. Phytother Res 2009;23:78–85.
[10] Choi YE, Yang DC, Kusano T, Sano H. Rapid and efficient *Agrobacterium*-mediated genetic transformation by plasmolyzing pretreatment of cotyledons in *Panax ginseng*. Plant Cell Rep 2001;20:616–21.
[11] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 -ΔΔC8 T method. Methods 2001;25:402–8.
[12] Lee MH, Jeong JH, Seo JW, Shin CG, Kim YS, In JG, Yang DC, Yi JS Choi YE. Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. Plant Cell Physiol 2004;45:976–84.
[13] Han JY, In JY, Kwon YS, Choi YE. Regulation of ginsenoside and phytosterol biosynthesis by RNA interferences of squalene epoxidase gene in *Panax ginseng*. Phytochemistry 2010;71:36–46.
[14] Furuya T, Yoshikawa T, Ishii T, Kajii K. Regulation of saponin production in callus cultures of *Panax ginseng*. Plant Med 1983;47:200–4.
[15] Lu MB, Wong HL, Teng WL. Effects of elicitation on the production of saponin in cell culture of *Panax ginseng*. Plant Cell Rep 2001;20:674–7.
[16] Yu KW, Gao W, Hahn EJ, Paek KY. Jasmonic acid improves ginsenoside accumulation in adventitious root culture of *Panax ginseng* C.A. Meyer. Biochemical Engineering J 2002;3596:1–5.
[17] Briskin DP. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Plant Physiol 2000;124:507–14.
[18] Nicol RW, Traquair JA, Bernards MA. Ginsenosides as host resistance factors in American ginseng (*Panax quinquefolius*). Can J Bot 2002;80:557–62.
[19] Katerere DR, Gray AI, Nash RJ, Waigh RD. Antimicrobial activity 1 of pentacyclic triterpenes isolated from African Combretaceae. Phytochemistry 2003;63:81–8.
[20] Mallvadhani UV, Mahapatra A, Raja SS, Manjula C. Antifeedant activity of some pentacyclic triterpene acids and their fatty acid ester analogues. J Agric Food Chem 2003;51:1952–5.
[21] Bernards MA, Yousef LF, Nicol RW. The allelopathic potential of ginsenosides. In: Inderjit, Mukerji KG, editors. Allelochemicals: Biological Control of Plant Pathogens and Diseases. Netherlands: Springer; 2006. p. 157–75.
[22] Sung WS, Lee DG. In vitro candidacidal action of Korean red ginseng saponins against candida albicans. Biol Pharm Bull 2008;31:139–43.
[23] Battinelli L, Mascellino MT, Martion MC, Lu M, Mazzanti G. Antimicrobial activity of ginsenosides. Pharin Pharmacol Comniun 1998;4:411–3.
[24] Zhao X, Gao J, Song C, Fang Q, Wang N, Zhao T, Liu D, Zhou Y. Fungal sensitivity to and enzymatic deglycosylation of ginsenosides. Phytochemistry 2012;78:65–71.
[25] Kang LJ, Choi YJ, Lee SG. Stimulation of TRAF6/TAK1 degradation and inhibition of JNK/AP-1 signalling by ginsenoside Rg3 attenuates hepatitis B virus replication. Int J Biochem Cell Biol 2013;45:2612–21.
[26] Lee MH, Han JY, Kim HJ, Kim YS, Huh GH, Choi YE. Dammarenediol-II production confers TMV tolerance in transgenic tobacco expressing *Panax ginseng* dammarenediol-II synthase. Plant Cell Physiol 2012;53:173–82.
[27] Lee MW. Studies on the root rot of ginseng (VII)-pathogenic microbial population in continuous culture and first-time culture of ginseng. Korean J Microbiol 1977;15:20–30.
[28] Yu YH, Ohh SH. Problems and present status of research on ginseng diseases in Korea. In: Bailey WG, Whitehead C, Proctor JTA, Kyle JT, editors. Proceeding of International Ginseng Conference. Vancouver (Canada): 1995. p. 120–30.