A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Over-expression and analysis of two endogenous UDP-glycosyltransferases genes in Korean wild ginseng (*Panax ginseng* C.A. Meyer)

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(Supervised by Professor Hyo-Yeon Lee)

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ABBREVIATIONS

NAA	Naphthalene acetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
2,4-D	2,4-Dichlorophenoxy-acetic acid
AR	Adventitious root derived from root itself
CR	Adventitious root derived from callus which induced from AR
NI	Root induction medium including NAA and IAA
ARIBA	AR roots induced by IBA root induction medium
CRIBA	CR roots induced by IBA root induction medium
ARNI	AR roots induced by NI root induction medium
CRNI	CR roots induced by NI root induction medium
HPLC	High performance liquid chromatography
PEMs	Proembryogenic mass
ACN	Acetonitrile
MS	Murashige & Skoog
FW	Fresh weight
DW	Dry weight
PPD	Protopanaxadiol
PPT	Protopanaxatriol

UV	Ultraviolet
PAT	Phosphinothricin acetyltransferase
PPT	Phosphinothricin
LB	Left border
RB	Right border
P35S	CaMV 35S promoter
RT-PCR	Reverse transcriptase polymerase chain reaction
SPSS	Statistical Package for the Social Sciences

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SUMMARY

Panax ginseng C.A Meyer (Korean wild ginseng) is an ancient and medicinal plant that wildly used all over the world. The most beneficial compound involved inside of ginseng plants is ginsenoside, with the high attentions have been paid to ginseng researches, more and more ginsenosides had been extracted and identified from ginseng plants. Up to now, more than 70 ginsenosides have been reported from natural and processed ginseng. In the meantime, various functional genes which are engaged in ginsenosides biosynthesis pathway come to be known, including UDP-glycosyltransferases (UGTs). UGTs play roles in the final step of ginsenoside synthesis, after glycosylate reactions catalyzed by UGTs result in variety of ginsenosides. In order to explore the function of UGTs in ginenosides biosynthesis pathway and increase the content of ginsenosides in ginseng adventitious roots, two endogenous UGTs genes (PgUGT74AE2 and PgUGT94Q2) were transformed to Korean wild ginseng by *Agrobacterium*-mediated transformation. The adventitious roots induction procedure was optimized by applying different rooting hormones, and induced adventitious roots directly from callus. Because different phenotypes were observed in different hormone rooting medium during culturing, morphological and histological analysis were applied for recording. In case of hormones and different resources give rise to the concentration of ginsenoside, high performance liquid chromatography (HPLC) was supposed for ginsenoside contents analysis for both wild-type and transgenic lines. Callus derived and adventitious roots derived roots rooting by IBA was observed increasing of total content of main ginsenosides (Re, Rg1, Rc, Rb1, Rb2, Rd), especially ginsenoside Rb2. As callus acting as the most suitable host for plant

genetic engineering, transformed callus derived roots rooting by IBA was supposed to produce transgenic roots in brief period. PgUGT74AE2 and PgUGT94Q2 transgenic lines generated within 6 month, including callus induction, transformation, roots induction from transformed callus. Transgenic candidates of PgUGT74AE2 and PgUGT94Q2 were selected by Immuno strip test (Agrastrip GMO, LL test Strips) as first confirmation. 9 lines of PgUGT74AE2 (74 L2, 74 L4, 74 3L2, 74 L2-2, 74 T2, 74 T3, 74 T5, 74 T6, 74 T7) and 7 lines of PgUGT94Q2 (94 L2, 94 T2, 94 T4, 94 T5, 94 T8, 94 T11, 94 T12) were screened from transgenic candidates. Total RNA was extracted from all transgenic lines, and cDNA synthesized from total RNA, cDNA used as template for RT-PCR analysis and *bar* gene confirmation. Genomic DNA extracted from all transgenic lines to performed southern blotting to analyze T-DNA region insertion copies. Incorporation with HPLC results of transgenic lines, PgUGT74AE2 transgenic lines have 4 lines (74 L2-2, 74 L2, 74 L4, 74 T2) and PgUGT94Q2 transgenic lines have 4 lines (94 L2, 94 T4, 94 T2, 94 T5) gained ginsenoside contents increasing compared with wild-type ginseng. And one unknown compound among these 8 transgenic lines showed content enhancement. Further analysis of unknown compound structure is in the management.

INTRODUCTION

Ginseng (*Panax ginseng* C.A. Meyer) is an important medicinal plant that is widely cultivated in eastern countries. The roots have been used as drugs for over 2000 years in oriental countries and also rapidly expanding in western countries as complementary and alternative medicine (Shim et al. 2009). The beneficial effects of ginseng are attributed to glycosylated tetracyclic triterpene compounds, also known as ginsenosides, are mainly produced in ginseng roots and reported mostly concentrated in adventitious roots (Han et al. 2013). Ginsenosides are mainly consisted of two parts named oleanane- and dammarene-type ginsenosides that generated at first cyclization of 2,3-oxidosqualene. And dammarene-type triterpenes are classified into two groups, protopanaxadiol- (PPD) and protopanaxatriol-type (PPT) ginsenosides, continuously with different glycosylation and result in various ginsenosides. More than 40 different ginsenosides producing various pharmacological effects have been identified in ginseng roots (Jung et al. 2014), and more than 70 ginsenosides have been investigated from ginseng plants including these involved in fresh ginseng (FG), suncured ginseng (SG) and red ginseng (RG) (Guo et al. 2014). Ginseng is a perennial plant that has a long production cycle (4-6 years) and greater than 3 years of juvenile period are required for producing seeds (Ahn et al. 1996; Choi et al. 1998), which has made the generation of superior genotypes by conventional breeding toughly to handle. Therefore, many attempts have been carried out to achieve more rapid ginseng plant regeneration procedure and increasing production of the ginsenosides. Biotechnological techniques have been applied in previous researches, such as classical tissue culture, bioreactor culture, *Agrobacterium*-mediated hairy root production, elicitors using in cell cultures and mutation

breeding by γ-irradiation (Yoshikawa et al. 1987; Bae et al. 2006; Kim et al. 2013).

P. ginseng is a difficult species to manipulate in vitro, however, the regeneration of ginseng has generally been accomplished by using somatic embryogenesis in callus derived from mature root tissues, callus derived from zygotic embryo (Lee et al. 1990), protoplast derived from callus, and cotyledons, aging callus produced numerous embryoids (Arya et al. 1991). Re-cultured of embryoids in MS solid medium supplemented with benzyladenine (6-BA) and gibberellic acid (GA_3) resulted in profuse plantlet regeneration (Chang et al. 1980). Metabolic engineering of *P. ginseng* thorough the transgenic adventitious root culture can be an important technique to upgrade medicinal value and produce efficient production of secondary metabolites from roots. Cotyledonary explants of ginseng zygotic embryos were co-cultured with *Agrobacterium tumefaciens* strain LBA4404 harboring binary vector carried with β-glucuronidase (GUS) gene, and transgenic plants were obtained by somatic embryogenesis (Lee et al. 1994). A rapid and efficient genetic transformation of *P. ginseng* by a plasmolyzing pretreatment of cotyledon explants was reported by Choi et al. (2001). However, more than 1 year is required to induce transgenic adventitious roots from regenerated transgenic plantlets via somatic embryogenesis. We are looking forward to find a new process to generate adventitious roots directly from calluses in brief period. Various hormone combinations were used to optimized ginseng adventitious roots induction and growth on the basis of previous researches.

At present, many novel UDP-glycosyltransferases (UGTs) genes which involved inside of ginsenosides biosynthesis have been reported by using advanced sequencing tools. It is reported that putative UDP-glycosyltransferases play a complex and important role in the

synthesis and metabolism of ginsenosides (Figure 2.)(Wang et al. 2011). Since the first UGT (UGTPg1) were reported by Yan et al. (2014), soon afterwards, consecutive UGTs and encoded genes were been coherented in intricate ginsenosides biosynthesis pathway. UGTPg1 from *P. ginseng* was characterized as a regio-specific enzyme which glycosylate the C20-OH position of PPD and also its derived ginsenosides (Yan et al. 2014). More currently, two UGTs named PgUGT74AE2 and PgUGT94Q2 from *P. ginseng* were characterized to catalyze the glycosylation of C3-OH of PPD to yield Rh2 and to elongate a glucose moiety of Rh2 and F2 to form Rg3 and Rd respectively (Jung et al. 2014; Wang et al. 2015; Wei et al. 2015). Not yet, no information is available about the UGTs transgenic lines by over-expressing of UGTs genes in *P. ginseng* plants. In this paper, we report an efficient procedure for producing transgenic lines of *P. ginseng* adventitious roots based on *Agrobacterium*-mediated transformation and root induction directly from wild-type ginseng somatic embryos. Two UDP-glycosyltransferases (UGTs) were supposed to be transferred into and over-expressed in Korean wild ginseng to enhance concentration of ginsenosides Rh2 and Rg3 or other possible related ginsenosides.

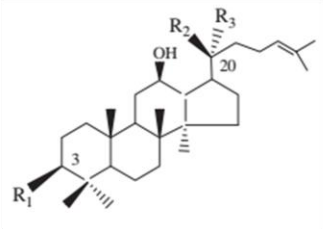
Ginsenosides: Triterpenoid saponins are secondary metabolites of isoprenoid compounds and mostly exist in higher plants. Ginsenosides are considered to be the primary components of the ginseng and have been verified mostly contained in adventitious roots. *P. ginseng* roots contain at least 4% ginsenosides by dry weight (Shibata et al. 2001). Six dammarane-type tetracyclic triterpenes (ginsenosides Rb1, Rb2, Rc, Rd, Re and Rg1) are reported as the major ginsenoside constituents (Figure 1.). Protopanaxadiol (PPD) is the reasonable substrate lied upstream of PgUGT74AE2 and PgUGT94Q2 encoded pathway. F2, S-Rh2, R-Rh2 and S-Rg3

are the possible products after PgUGT74AE2 and PgUGT94Q2 encoded glycosylation according to published paper (Jung et al. 2014). It has been reported that ginsenosides Rh2 and Rg3 are potent medicinal agents for inducing tumor cell apoptosis (Min et al. 2006; Park et al. 1997), inhibiting tumor cell proliferation (Kim et al. 2004). Rh2 and Rg3 are also potentially effective candidates for preventing metabolic disorders of human beings, such as diabetes and obesity, via activating the AMPK signaling pathway (Hwang et al. 2007; Park et al. 2008). Indeed, as ginsenosides Rh2 and Rg3 are the versatile compound value of infrequent bioactivities, a huge demand has been existed to manufacturing them for commercial use. Whereas, their contents inside of ginseng are extremely low in natural ginseng, the contents of Rh2 and Rg3 can be increased markedly to 0.001% and 0.015%, respectively, in red ginseng, which the production process performed by steaming and drying harvested ginseng root (Shibata et al. 2001).

UDP-glycosyltransferases: Glycosylation of natural products in plants is catalyzed by glycosyltransferases (GTs), leading to changes in hydrophilicity, stability, and subcellular localization, and thus the chemical properties and bioactivity of the natural products (Bowles et al. 2006). Enzymes belonging to the multigene families of oxidosqualenecyclases, and UDP-glycosyltransferases are key players in biosynthesis of plant triterpenoid saponins. (Augustin et al. 2011). GTs are members of a multigene superfamily that catalyzes the transfer of sugar moieties to specific acceptors. The GTs that take usage of uridine diphosphate (UDP)-activated sugar molecules as donors are referred as UDP-glycosyltransferases (UGTs) (Barvkar et al. 2012; Ross et al. 2001). Nevertheless, only a limited number of UGTs characterized to glycosylate triterpenoid aglycones have been

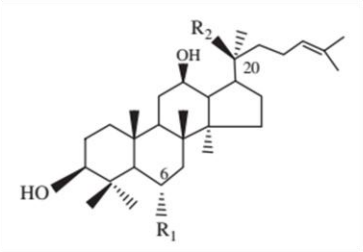
exposed from plants, such as *Medicago truncatula* (Achnine et al. 2005), *Saponaria vaccaria* (Meesapyodsuk et al. 2007), *Barbarea vulgaris* (Augustin et al. 2012), *Glycine max* (Shibuya et al. 2010), and *P. ginseng* (Yan et al. 2014; Wang et al. 2015). Two endogenous genes PgUGT74AE2 and PgUGT94Q2 from *P. ginseng* were characterized to catalyze the glycosylation of C3-OH of PPD to yield Rh2 and to elongate a glucose moiety of Rh2 and F2 to form Rg3 and Rd respectively, and convert reactions have been demonstrated in yeast system (Jung et al. 2014).

Protopanaxadiol (PPD) type ginsenosides.
glc: b-D-glucopyranosyl
arap: a-L-arabinopyranosyl
araf: L-arabinofuranosyl.



Compound	Ginsenoside	R1	R2	R3
1	PPD(S)	OH	OH	CH3
2	Rg3(S)	O-glc(2→1)glc	OH	CH3
3	Rg3(R)	O-glc(2→1)glc	CH3	OH
4	Rh2(S)	O-glc	OH	CH3
5	Rh2(R)	O-glc	CH3	OH
6	Rb1	O-glc(2→1)glc	O-glc(6→1)glc	CH3
7	Rc	O-glc(2→1)glc	O-glc(6→1)araf	CH3
8	Rb2	O-glc(2→1)glc	O-glc(6→1)arap	CH3
9	Rd	O-glc(2→1)glc	O-glc	CH3
10	F2	O-glc	O-glc	CH3

Protopanaxatriol (PPT) type ginsenosides.
glc: b-D-glucopyranosyl
rha: a-L-rhamnopyranosyl.



Compound	Ginsenoside	R1	R2
11	Rg1	*O*-glc	*O*-glc
12	Re	*O*-glc(2→1)rha	*O*-glc

Figure 1. Structures and accurate masses of ginsenosides.

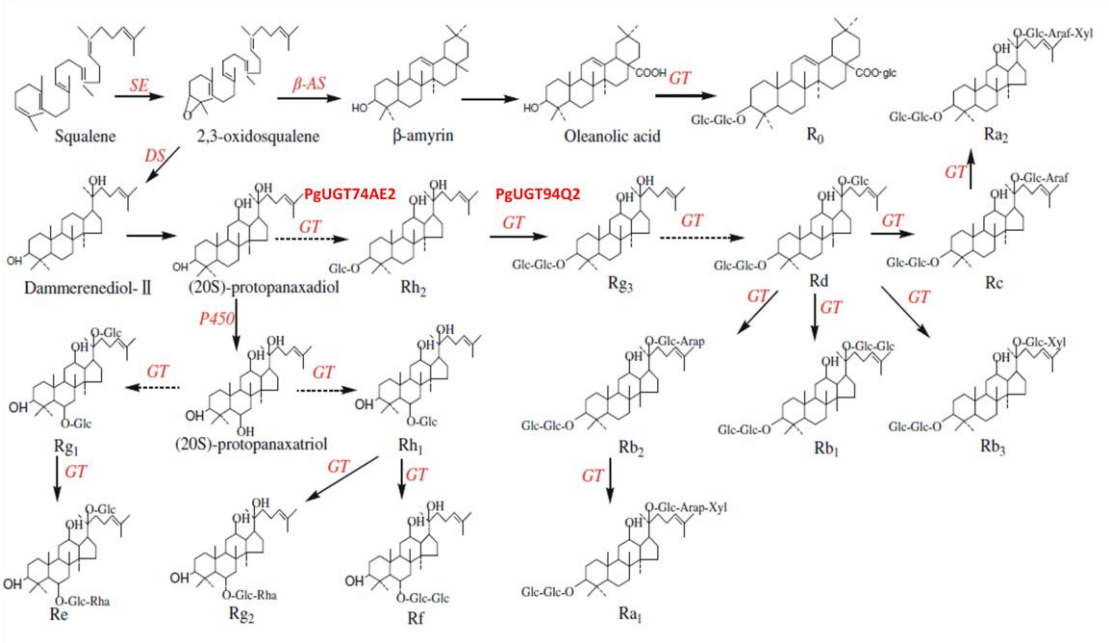


Figure 2. Putative ginsenosides biosynthesis pathway in *P. ginseng*. (Wang et al. 2011)

IPP, isopentenyl diphosphate; β-AS, β-amyrin synthase; DS, dammarenediol-II synthase; GT, UDP-glycosyltransferases.

MATERIALS AND METHODS

Callus induction and selection

Adventitious roots derived from Korean wild ginseng were provided by Sunchon National University, Sunchon, Korea. The adventitious roots were generated as described previously and have been maintained in our laboratory for over 10 years (Zhang et al. 2011). Calluses were induced from fresh adventitious roots remained on MS solid medium with 2mg/L NAA and 0.25mg/L IAA in plates culture. Wild-type adventitious roots were sectioned into 10 mm in length and were placed on Murashige and Skoog (MS) solid medium supplemented with 0.5mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.3mg/L kinetin, and 3% sucrose and media were solidified with 0.3% Gelrite (Zhang et al. 2014). All media were adjusted to pH 5.8 prior to autoclaving. Ten pieces of adventitious roots were placed on each petri dish. Callus formation was observed after 4 weeks of culture. The induced callus was sub-cultured at 3-wk intervals on the same medium for induction of embryogenic callus and maintenance. After 2 months of culture, we selected the compact and strong pro-embryogenic masses and used for somatic embryos induction. Somatic embryos induction medium was MS medium supplemented with 0.5mg/L 2,4-D. Induced somatic embryos were inoculated on MS solid medium for enlarge cultivation with 0.5mg/L 2,4-D every 3 weeks. All media were adjusted to pH 5.8 prior to autoclaving. All cultures were incubated at 23 ± 2 ℃ in the dark.

Optimization of adventitious roots induction procedure

Wild-type ginseng adventitious roots were used to induce calluses, from calluses to form somatic embryos and then induce roots from somatic embryos directly. We applied two

different roots induction medium according to previous research paper (Zhang et al. 2014 and Huang et al. 2010), and measured the root induction efficiency of both roots and calluses while the culturing procedure. Somatic embryos and adventitious roots were inoculated onto MS solid media containing 5.0 mg/L indole-3-butric acid (IBA), 30 g/L sucrose and 0.3% gelrite (IBA medium) , and MS solid medium containing 2 mg/L NAA, 0.25mg/L IAA and 0.3% gelrite (NI medium). Also hormone-free MS solid medium was used for control. Three replicates were prepared for each treatment. Roots performed further development in conical flask containing 50ml liquid MS medium supplemented with 5.0 mg/L IBA or 2 mg/L NAA, 0.25mg/L IAA, respectively, on a rotary shaker (120 rpm) at 23 ± 2 ℃ and sub-cultured every 4 weeks. Plates culturing of ginseng roots were implemented in dark, then further culturing in flasks were maintained under a 16-h (light)/8-h (dark) photoperiod with light supplied by white fluorescent tubes at an intensity of 30 μmol m^{-2} s^{-1}.

Morphologic and histological analyses of CR and AR

Comparison of two different roots induction medium was showed in morphological differences, such as growth ratio, secondary root number, length and diameter. Fresh weight (FW) and dry weight (DW) were measured after two months culture either on plates or in flasks culture. Harvest roots was rinsed by tap water once and then put on tissue paper for 10 minutes and blotting with tissue paper to remove residual water, straight after, measure about FW of roots. And DW was recorded after fresh roots were dried to constant weight. Root growth ratio was calculated as following formula (Yu et al. 2002).

Growth ratio= Harvest DW (g)/Inoculated DW (g)

The length of secondary roots was measured by calipers. The number of occurred secondary roots was counted from main roots body. The diameters of secondary roots were measured from microscope and Nikon NIS-Elements system by using measure scale. Newly developed adventitious roots of all treatments were selected for checking callus formation ability. Callus induction medium referred to callus induction and selection section.

Gene cloning and vectors construction

Total RNA was extracted from wild-type ginseng adventitious roots by using Trizol (Invitrogen, Carlsbad, CA, USA) reagent, and cDNA synthesized from total RNA by using M-MLV Reverse Transcriptase kit (Promega, Seoul, Korea). Synthesized cDNA used as template and gateway cloning method used for PgUGT74AE2 (JX898529) and PgUGT94Q2 (JX898530) genes cloning. Specific primers were designed according to registered sequences on NCBI (Table 1.). *Pfu* DNA Polymerase (Promega, Seoul, Korea) is supposed to generate blunt ends and yield high fidelity PCR products. Then PCR products ligated into pENTR vector according to pENTR™/D-TOPO® cloning kit protocol (Invitrogen, Carlsbad, CA, USA). Ligated pENTR vector was transformed to competent TOP10 E. *coli* cells. Positive transformant was selected and isolated plasmid DNA, and then confirm the sequence. pENTR vector contains target genes sub-cloned into destination vectors (pB2GW7,0) by LR recombination (Invitrogen, Carlsbad, CA, USA). LR reaction mixture transformed to competent TOP 10 E. *coli* cells, spread on LB solid medium contained 50mg/L spectinomycin. Harvested positive transformant and extracted plasmid DNA then transferred to *Agrobecterium* EHA105 competent cells, and spread on YEP solid medium contained 25

mg/L rifampicin and 50mg/L spectinomycin. Harvested positive transformant make *Agrobacterium* cell stocks which contained transgene and used for plant transformation.

***Agrobacterium*-mediated transformation**

Selected calluses were pre-treated in hormone-free MS liquid medium containing 30g/L sucrose for 1 day. *A. tumefaciens* EHA105 cells cultured in YEP medium with 25mg/L rifampicin and 50mg/L spectinomycin for 24h at 28℃, 180rpm. The pre-treated calluses were immersed in the suspension of *A. tumefaciens* EHA105 carried with destination vectors in hormone-free MS liquid medium containing 100 mg/L acetosyringone. After 24h cultured in dark on a gyratory shaker, the calluses were placed on sterilized filter paper for 10 min to remove extra *Agrobacterium* cells, and then cultured on hormone-free MS solid medium containing 100 mg/L acetosyringone, 3% sucrose, 0.3% gelrite at 23±2℃ in the dark 3 days for co-culture. Thereafter, the calluses were cultured on MS medium with 3% sucrose and 600 mg/L cefotaxime 3 weeks for elimination, and then transferred to selection MS solid medium with 600 mg/L cefotaxime, 1mg/L PPT，5mg/L IBA and 0.3% gelrite. All media were adjusted to pH 5.8 prior to being autoclaved at 120℃ for 15 min.

Production of transgenic roots

Transformed calluses were inoculated onto MS solid media containing 5.0 mg/L indole-3-butric acid (IBA), 600mg/L cefotaxime, 1mg/L PPT, 30 g/L sucrose and 0.3% gelrite, sub-cultured every 3 weeks. Newly developed roots from transformed calluses were selected and cut from resourced calluses to perform further development on new solid root induction medium, and amplified in conical flasks containing 50ml liquid MS medium supplemented

with 5.0 mg/L IBA, 600mg/L cefotaxime, 1mg/L PPT on a rotary shaker (120 rpm) at 23 ± 2 ℃ and sub-cultured every 4 weeks.

Genotype analysis of transgenic candidates

Newly developed roots from transfected calluses were supposed as transgenic candidates. Immuno strip test (Agrastrip GMO, LL test Strips) used for the most convenient method to confirmed genetically modified organism (GMO). For the first confirmation of transgenic lines, we chose the transgenic candidates length reached about 2cm, half kept for strip test, and another half remained on root induction medium for further development. Transgenic lines would get two bands on test strips, while wild-type lines would gain only one band, and under bands indicated on strips are the indication of *bar* gene encoded protein, PAT (phosphinothricin acetyltransferase) protein. With the first step confirmation, PgUGT74AE2 transgenic lines and PgUGT94Q2 transgenic lines were selected and amplified in conical flask containing 50ml liquid MS medium supplemented with 5.0 mg/L IBA, 600mg/L cefotaxime, 1mg/L PPT on a rotary shaker (120 rpm) at 23 ± 2 ℃ and sub-cultured every 6 weeks. Each line sampled with liquid nitrogen grounding in mortar, and collected samples divided to extract total RNA and genomic DNA. Total RNA then performed to do cDNA synthesis, cDNA of each lines was used as template to do RT-PCR (Reverse transcript-polymerase chain reaction) to check relative expression of transgene (Table 1), genomic DNA from transgenic lines was used for Southern blotting to analyze T-DNA integration and to confirm the copy number.

Extraction and determination of crude saponin

Extraction method of ginsenosides from ginseng roots followed and modified from the method of Jin et al. (2012) and Su et al. (2016). Ginseng samples of wild-type and transgenic lines were accurately weighed (approximately 0.5 g) and ultrasonic-extracted with 2.0 ml of methanol in an ultrasonic water bath for 15 min at the temperature of 40℃, each sample repeats ultrasonic extraction for 3 times and eventually get 6 ml volume in total. The extracts were transferred into pear-shaped flasks and evaporated to dryness at 40℃ with a vacuum rotary evaporator. Then, the residue was dissolved in 1.0 ml of methanol and filtered by a 0.22 μm PTFE syringe filter before HPLC analysis (Figure 3).

HPLC analysis of wild-type and transgenic lines

For ginsenosides assay of wild-type and transgenic lines, the HPLC (High Performance Liquid Chromatography) conditions followed Jung et al. (2014) and made some modifications (Table 2). HPLC running conditions showed as following table (Table 3.). All the solvents were belonged to HPLC grades. Ginsenoside standards (Re, Rg1, Rb1, Rc, Rb2, Rd, Rh2 and Rg3) were purchased from SHANGHAI ZZBIO CO., LTD. An ODS C18 column (Shiseido, Japan) was selected as stationary phase, mobile phase including Solvent (A): Acetonitrile, Solvent (B): Distilled water. Ginsenoside standard solutions were prepared in 100% HPLC grade methanol. A series gradient concentration (50ppm, 100ppm, 400ppm) of each standard were tested for calibration curve.

Analysis of ginsenoside contents was performed according to Son et al. (1999a) and Yu et al. (2000). The total ginsenoside content was calculated as the sum of individual ginsenoside

fractions.

The ginsenoside content of ginseng adventitious roots was calculated as:

(GC: ginsenoside content; SGC: sample ginsenoside concentration from HPLC; SV: sample volume; AR: adventitious root derived from root itself; CR: adventitious root derived from callus which induced from AR)

$$GC(mg\ \ g^{-1}) = \frac{SGC(\ fromHPLC)(mg\ \ g^{-1})\ \ X\ \ SV(l)}{\mathrm{AR\ or\ C}R(g)}$$

Statistical analysis

Statistical analysis achieved by using SPSS (Statistical Product and Service Solutions) system, IBM SPSS Statistics version 22.0. Mean and standard errors were measured throughout datas and statistical significance between the mean values was assessed by Duncan's multiple range tests. A probability of $P < 0.05$ was considered as significantly.

Table 1. Primers sets used in experiments.

PCR types	Genes	Oligo sequences (5'->3')
Gateway cloning	PgUGT74AE2	FW:CACCATGGATAACCAAAATGGTAGA RE: CTATTGTTCATCTTTCTTCTTCTT
	PgUGT94Q2	FW:CACCATGCTGAGCAAAACTCACATT RE:TCAGGAGGACACAAGCTTTGAAAT
RT-PCR	PgUGT74AE2	FW: CGAACCCGAACGTACAAAGCT RE: CCCAAACTCAATGCCTCAACC
	PgUGT94Q2	FW: TCAAAGGGTAGGAGACAGAGG RE: GCTTACCATTCAAAGGCTGAT
	18s rRNA	FW: ATGATAACTCGACGGATCGC RE: CCTCCAATGGATCCTCGTTA
Probe	PgUGT74AE2	FW: GCTCGGTAGTCTACGCCTCAT RE: CTATTGTTCATCTTTCTTCTTCTT
	PgUGT94Q2	FW: GGCTGAATCTACAGTGGTGTT RE:TCAGGAGGACACAAGCTTTGAAAT
	Bar	FW: AAGTCCAGCTGCCAGAAACCCAC RE: GTCTGCACCATCGTCAACCACTA

Table 2. HPLC conditions for ginsenoside analysis.

Parameter	**Condition**
Instruments	Shimadzu HPLC system (DGU-20A, LC-20AD, SIL-20A, CTO-20A, SPD-M20A, CBM-20A)
Column	Capcell-pak C18 MG (4.6 × 250 mm) column, 5μm (Shiseido, Japan)
Mobile phase	Distilled water and Acetonitrile
Flow rate	1 mL/min
Detector	Wavelength: 203 nm (PDA)
Scan wavelength	190 - 400 nm
Column temperature	35℃
Sample injection	20 μL
Run time	28 min

Table 3. Mobile phase of HPLC gradient conditions for ginsenoside analysis.

Retention time (min)	Solvent (A)	Solvent (B)
0	32	68
8	65	35
12	100	0
20	100	0
20.1	32	68
28	32	68

Solvent (A): Acetonitrile, Solvent (B): Distilled water.

Table 4. Media composition for ginseng tissue culture.

Medium		**Composition**
Callus induction		MS medium, 30 g/L sucrose, 0.5 mg/L 2,4-D, 0.3 mg/L kinetin, 3 g/L Gelrite, pH 5.8.
Callus growth		MS medium, 30 g/L sucrose, 0.5 mg/L 2,4-D, 0.3 mg/L kinetin, 3 g/L Gelrite , pH 5.8.
Somatic embryos induction		MS medium, 30 g/L sucrose, 0.5 mg/L 2,4-D, 3 g/L Gelrite , pH 5.8.
Root induction	IBA	MS medium, 30 g/L sucrose, 3 g/L Gelrite , 5mg/L IBA, pH 5.8.
	NI	MS medium, 30 g/L sucrose, 3 g/L Gelrite , 2mg/L NAA, 0.25mg/L IAA, pH 5.8.

Table 5. Media compositions used for transformation.

Medium	**Composition**
Callus induction	MS medium with 30 g/L sucrose, 0.5 mg/L 2,4-D, 0.3 mg/L kinetin, 3 g/L Gelrite , pH 5.8.
Callus growth	MS medium, 30 g/L sucrose, 0.5 mg/L 2,4-D, 0.3 mg/L kinetin, 3 g/L Gelrite , pH 5.8.
Somatic embryos induction	MS medium, 30 g/L sucrose, 0.5 mg/L 2,4-D, 3 g/L Gelrite , pH 5.8.
Agrobacterium culture	Yep medium, 50 mg/L spectinomycin, 25 mg/L rifampicin, pH 7.0.
Agrobacterium resuspension	MS medium, 100 mg/L acetosyringone, 30 g/L sucrose, pH 5.8.
Co-cultivation	MS medium, 30 g/L sucrose, 3 g/L Gelrite , 100 mg/L acetosyringone, pH 5.8.
Elimination	MS medium, 30 g/L sucrose, 3 g/L Gelrite , 300 mg/L cefotaxime, pH 5.8.
Selection	MS medium, 30 g/L sucrose, 3 g/L Gelrite , 0.5 mg/L 2,4-D, 0.3 mg/L kinetin, 300 mg/L cefotaxime, 3 mg/L PPT, pH 5.8.
Shoot induction	MS medium, 30 g/L sucrose, 3 g/L Gelrite , 300 mg/L cefotaxime, 3 mg/L PPT, 5 mg/L GA_3, pH 5.8.
Root induction	MS medium, 30 g/L sucrose, 3 g/L Gelrite , 300 mg/L cefotaxime, 5mg/L IBA and 1 mg/L PPT, pH 5.8.

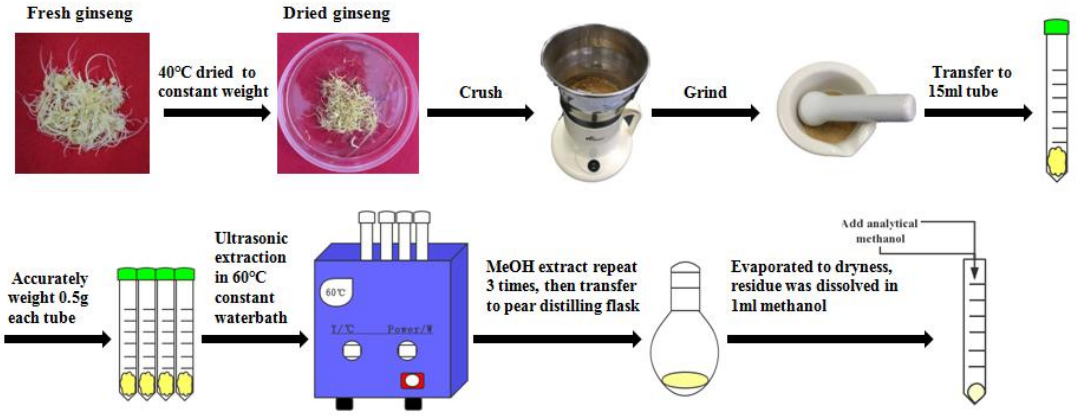


Figure 3. Schematic extraction procedure of crude saponin.

RESULTS

Callus induction and selection

During the culture procedure, root segments started swelling after 2 weeks culture, proembryogenic mass (PEMs) produced firstly on the MS solid medium with 2,4-D and kinetin after 1 month culturing. Then PEMs were divided to pieces, around 0.5mm in diameter for following somatic embryos induction, kinetin was removed and white and strong somatic embryos gained on somatic embryos induction medium, somatic embryo calluses were used for *Agrobacterium*-mediated transformation and adventitious roots induction host.

Optimization of roots induction procedure and comparison between AR and CR

Callus-derived roots (CR) induced from compact and strong callus masses within 1 month, identical phenomenon were discovered in both roots derived adventitious roots (AR) and callus derived adventitious roots (CR) in roots induction procedure though incubating with different rooting hormone (Figure 4). Measurements of newly developed adventitious roots inducing efficiency on different rooting medium were recorded after 6 weeks of rooting culture, also first derived roots segments from callus were tested for rooting ability. IBA rooting medium gained significant enhancements for callus, CR and AR in secondary roots inducing number (Table 6), behaved similar performances in diameter of secondary roots among each treatment, but uncontemplated with extraordinary shorter length of secondary roots than NI rooting medium (Figure 4). As for growth ratio of adventitious roots development, IBA treatments behaved exemplary both in flask shaking liquid culture and plate solid culture (Figure 5). In the case of callus formation ability, adventitious roots rooting

by NAA and IAA medium presented higher callus formation frequency than IBA rooting medium (Table 7).

Production of transgenic roots and genotype analysis

Transgenic candidates generated from somatic embryos after 2 month sub-culturing on roots induction medium, MS solid medium with 5mg/L IBA, PPT 1mg/L and 300mg/L cefotaxime. *Bar* gene confirmation and RT-PCR (Reverse transcript-polymerase chain reaction) analysis of and PgUGT74AE2 and PgUGT94Q2 transgenic lines (CRIBA is supposed as wild-type control) were shown in Figure 7 and Figure 9. There had 5 lines (T2, T4, T5, T8, L2) of PgUGT94Q2 transgenic lines and 8 (T2, T6, L2-2, T7, L2, L4, T5, 3L2) of PgUGT74AE2 transgenic lines gained higher relative expression level while compared with wild-type control. Incorporation with southern blot results, 7 lines (T2, T4, T5, T8, T11, T12, L2) of PgUGT94Q2 transgenic lines appeared 4, 2, 1, 1, 3, 1 and 2 bands respectively, 9 lines (L2, L4, T2, T7, T3, T5, T6, L2-2, 3L2) of PgUGT74AE2 transgenic lines appeared 1, 3, 3, 1, 7, 2, 1, 1, 2 bands respectively. Single copy of transgenic lines (PgUGT94Q2 T5, PgUGT74AE2 L2-2, PgUGT74AE2 L2) exerted high relative expression, two copies (PgUGT94Q2 T4, PgUGT94Q2 L2), triple copies (PgUGT74AE2 L4, PgUGT74AE2 T2) and quadruple copies (PgUGT94Q2 T2) also showed up high relative expression, while single copy of PgUGT94Q2 T12 and triple copies of PgUGT94Q2 T11 acted out low relative expression, 7 copies of PgUGT74AE2 acted out low relative expression (Figure 7-10). HPLC analysis of all transgenic lines was carried out to check T-DNA insert effect and ginsenosides variation.

HPLC analysis of wild-type and transgenic lines

CRIBA treatment showed highest concentration of ginsenosides among all 4 treatments (ARIBA, CRIBA, ARNI and CRNI) (Figure 10-11). CRIBA was used as wild-type control and contrasted with transgenic lines, PgUGT74AE2 transgenic groups have 4 lines (74 L2-2, 74 L2, 74 L4, 74 T2) and PgUGT94Q2 transgenic groups have 4 lines (94 L2, 94 T4, 94 T2, 94 T5) gained ginsenosides contents increasing. And the content of one unknown compound increased in these 8 transgenic lines (Figure 12-15). Transgenic line PgUGT74AE2 T2 showed the highest ginsenosides concentration among wild-type control and other PgUGT74AE2 transgenic lines, and 1.4-fold higher than that of wild-type ginseng. Transgenic line PgUGT94Q2 T2 showed the highest ginsenosides concentration among wild-type control and other PgUGT94Q2 transgenic line, and 1.3-fold higher than that of wild-type ginseng.

Table 6. Comparison of different medium for ginseng adventitious roots induction from calluses and adventitious roots[1)]

Explant types	Induction medium	Number of inoculated explants	Number of induced roots from each explants	Frequency of roots formation （%）
Callus	Non	30	0.67 ±1.27^{e}	33 ±22.14
	NI	30	16.53 ±2.98^{cd}	100
	IBA	30	20.77 ±4.72^{bc}	100
Adventitious root	Non	30	1.57 ±1.81^{e}	49 ±21.32
	NI	30	13.3 ±2.9^{de}	100
	IBA	30	24.03 ±6.14^{a}	100
Callus-derived adventitious root	Non	30	0.37 ±0.81^{e}	61 ±35.42
	NI	30	12.07 ±3.22^{de}	100
	IBA	30	24.1 ±5.36^{a}	100

1) Data were collected after 6 weeks of culture. The results represent the means standard error obtained from three repeats. Different corresponding letters within a column are significant different at $p < 0.05$ by Duncan's multiple range test.

Non: Hormone-free.

NI: MS solid medium supplemented with 2mg/L NAA, 0.25mg/L IAA and 0.3% Gelrite.

IBA: MS solid medium supplemented with 5mg/L IBA and 0.3% Gelrite.

Table 7. Frequency of callus formation of ginseng adventitious roots from AR and CR groups[1)]

Roots types	Number of root explants	Number of root explants forming callus	Callus induction frequency（%）
ARNI	30	15.67±1.53[a]	47.78±5.09
ARIBA	30	5.67±2.52[b]	20±5.78
CRNI	30	14.33±1.53[a]	52.22±5.09
CRIBA	30	6±1.73[b]	18.89±8.39

1) Data were collected after 6 weeks of culture. The results represent the means standard error obtained from three repeats. Different corresponding letters within a column are significant different at $p < 0.05$ by Duncan's multiple range tests.

ARNI: Adventitious roots induced by NI medium and induced callus on callus induction medium.

ARIBA: Adventitious roots induced by IBA medium and induced callus on callus induction medium.

CRNI: Callus-derived adventitious roots induced by NI medium and induced callus on callus induction medium

CRIBA: Callus-derived adventitious roots induced by IBA medium and induced callus on callus induction medium.

Callus induction medium: MS solid medium supplemented with 0.5mg/L 2,4-D, 0.3mg/L kinetin, 30g/L sucrose and 0.3% Gelrite.

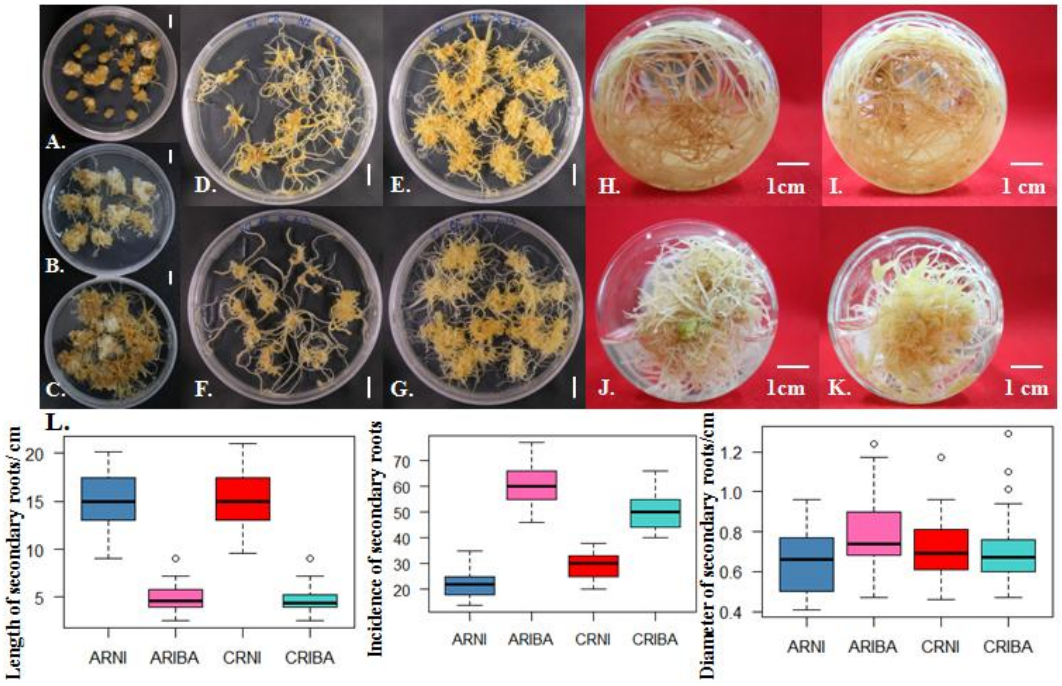


Figure 4. Morphological comparison between CR and AR cultured on different rooting medium. A~G, incubated on MS solid medium, solidified with 0.3% Gelrite. H~K, incubated in MS liquid medium with rotary shaking (120 rpm). A, Calluses cultured on hormone-free medium; B, Calluses cultured on NI medium; C, Calluses cultured on IBA medium; D, H, Callus-derived roots induced by NI medium; E, J, Callus-derived roots induced by IBA medium; F, I, Adventitious roots induced by NI medium; G, K, Adventitious roots induced by IBA medium; L, Comparison of length, incidence and diameter of secondary roots in flask shaking culture among each treatment, data analyzed by R studio version 1.0.44. ARNI, Adventitious roots cultured in NI medium; ARIBA, Adventitious roots cultured in IBA medium; CRNI, Callus-derived roots cultured in NI medium; CRIBA, Callus-derived roots cultured in IBA medium. *Bar* 1cm.

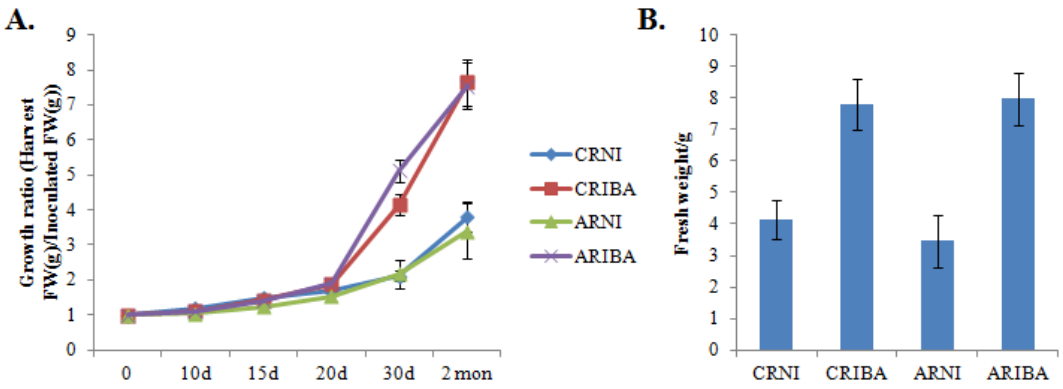


Figure 5. Effect of plant hormones on growth of ginseng adventitious roots. A, Growth ratio of adventitious roots in the flask culture. Original weight is 1g for all types of adventitious roots. B, Fresh weight of adventitious roots in solid root induction medium. Each plate incubated with 10 root segments originally. ARNI: Adventitious roots cultured on NI medium; ARIBA: Adventitious roots cultured on IBA medium; CRNI: Callus-derived adventitious roots cultured on NI medium; CRIBA: Callus-derived adventitious roots cultured on IBA medium.

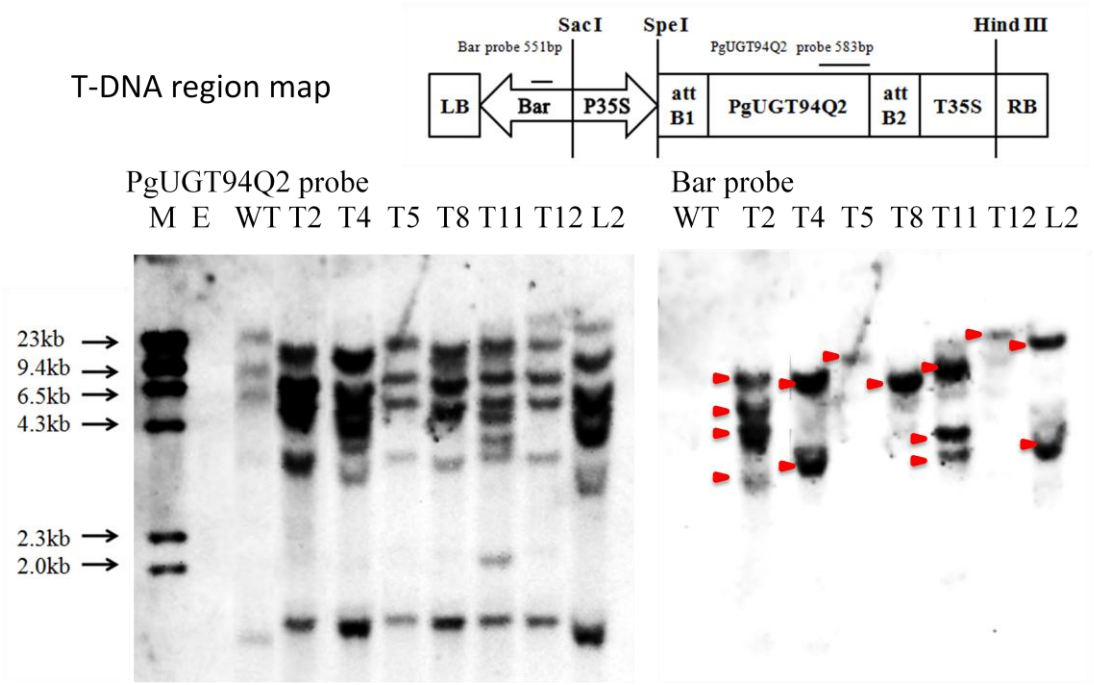


Figure 6. Southern blot analysis of PgUGT94Q2 transgenic lines.

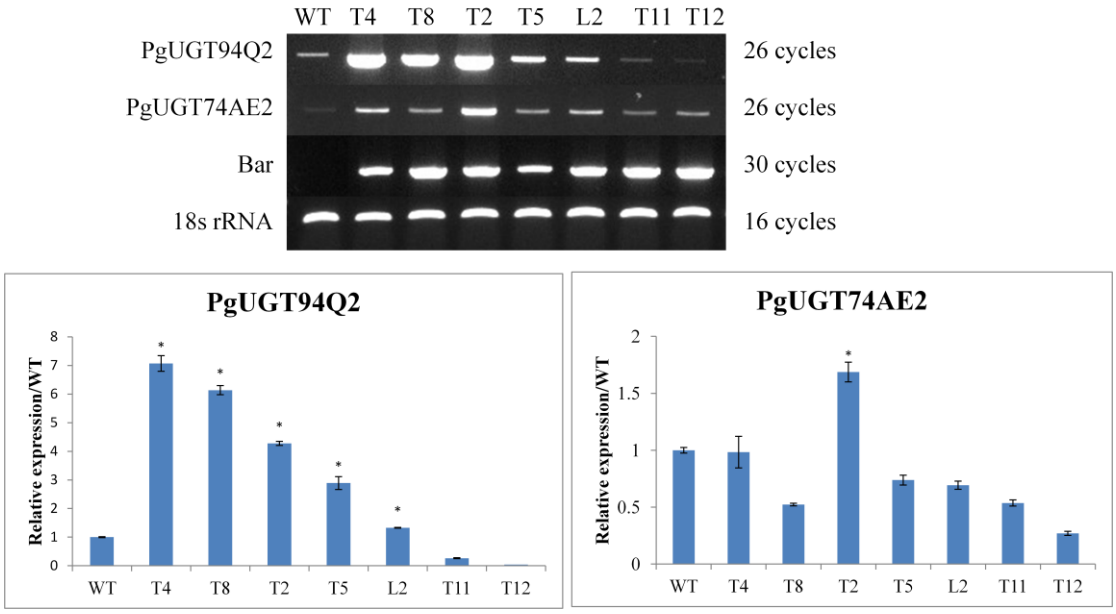


Figure 7. RT-PCR analysis of PgUGT94Q2 over-expression lines.

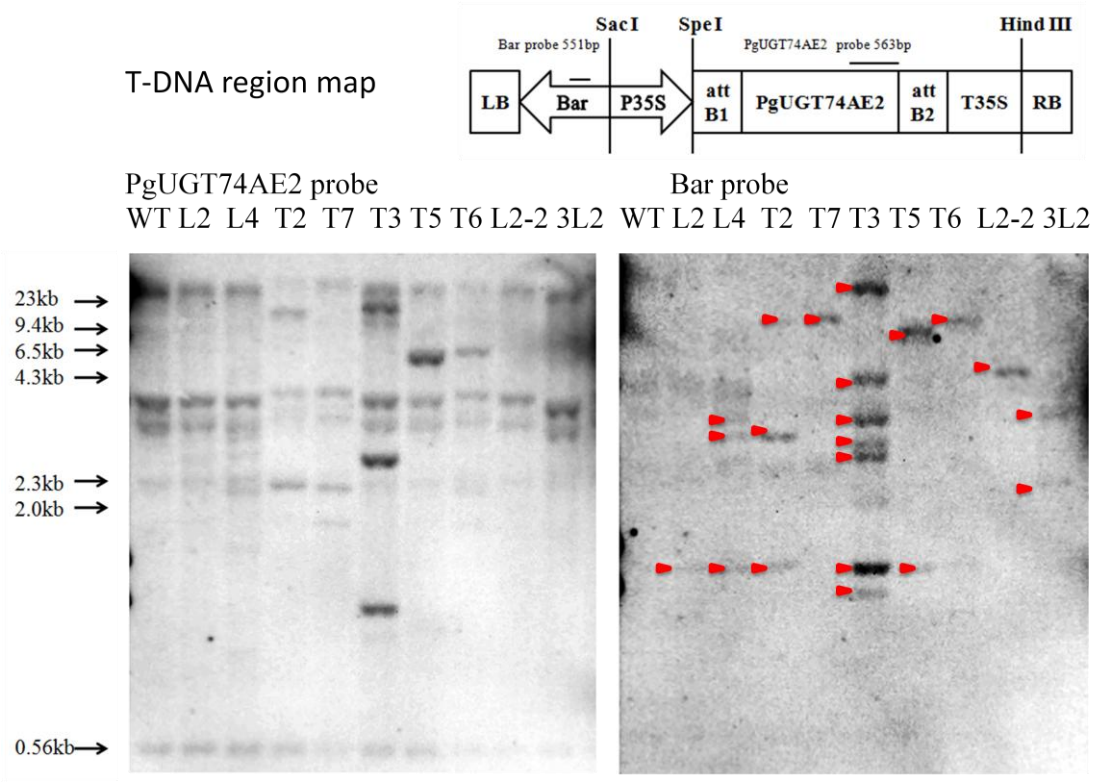


Figure 8. Southern blot analysis of PgUGT74AE2 transgenic lines.

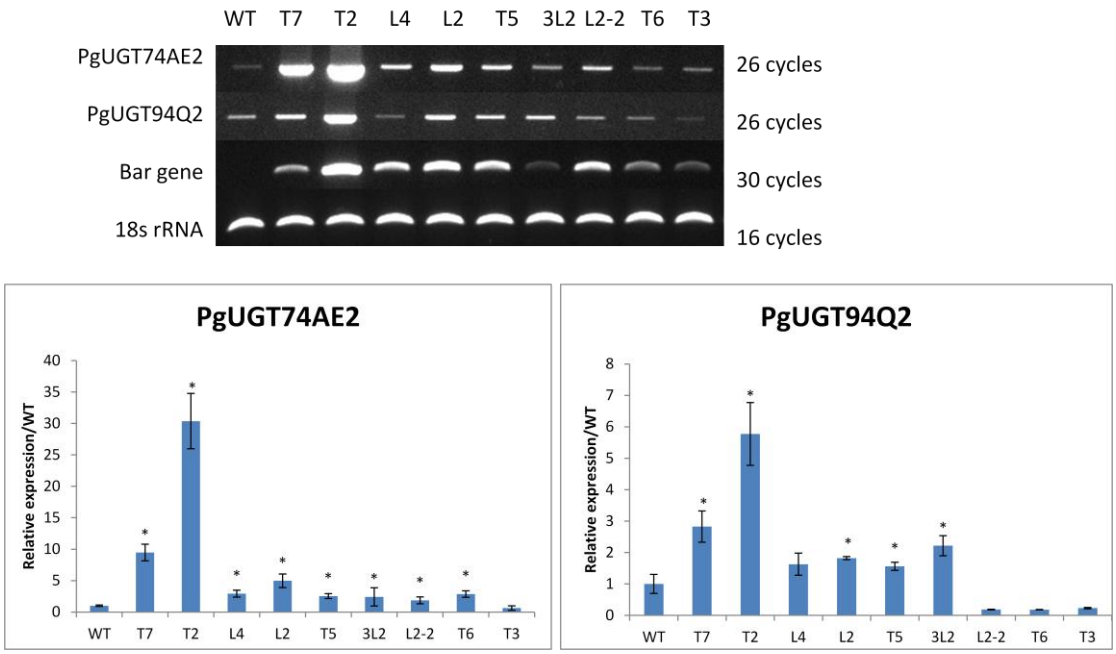


Figure 9. RT-PCR analysis of PgUGT74AE2 over-expression lines.

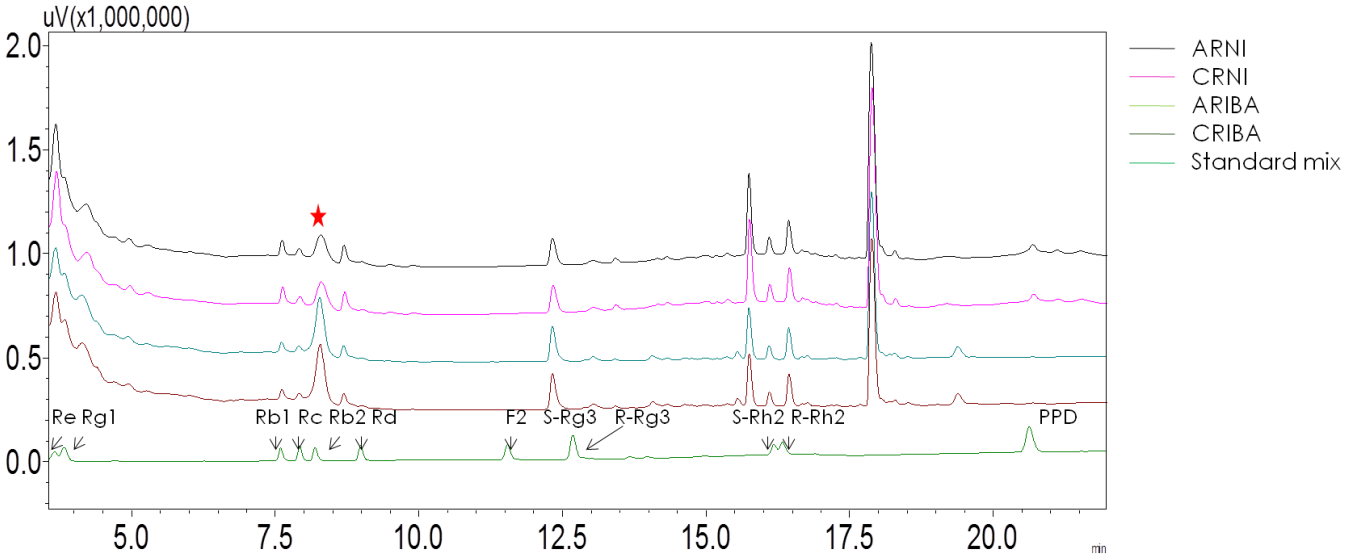


Figure10. Comparison of ginsenoside contents of AR and CR that induced by NI (NAA and IAA) and IBA.

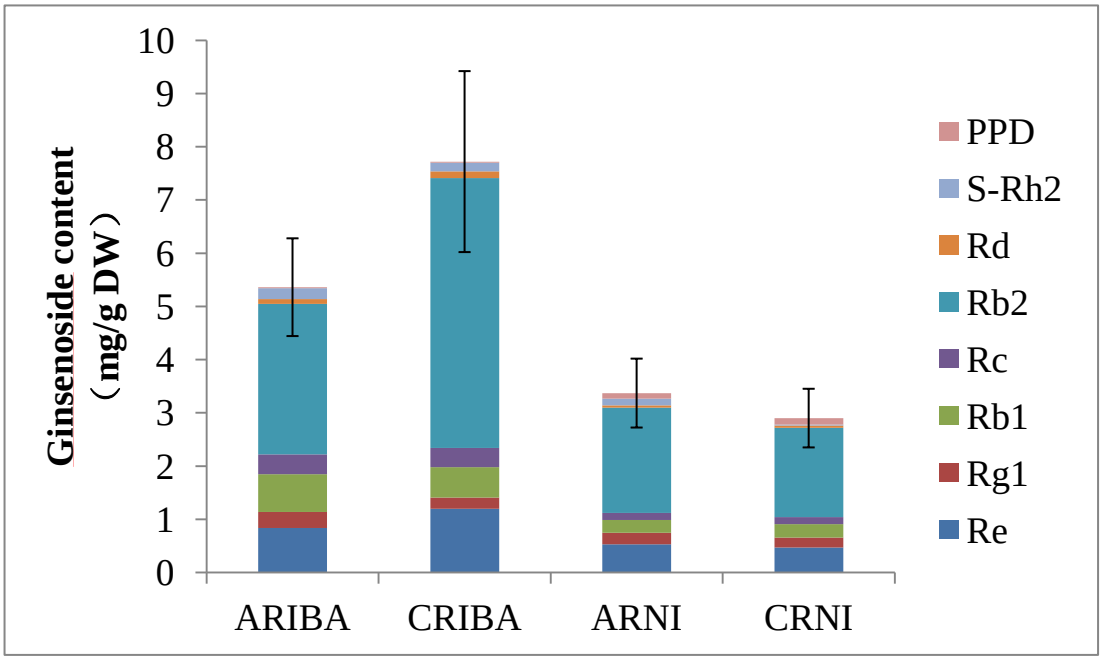


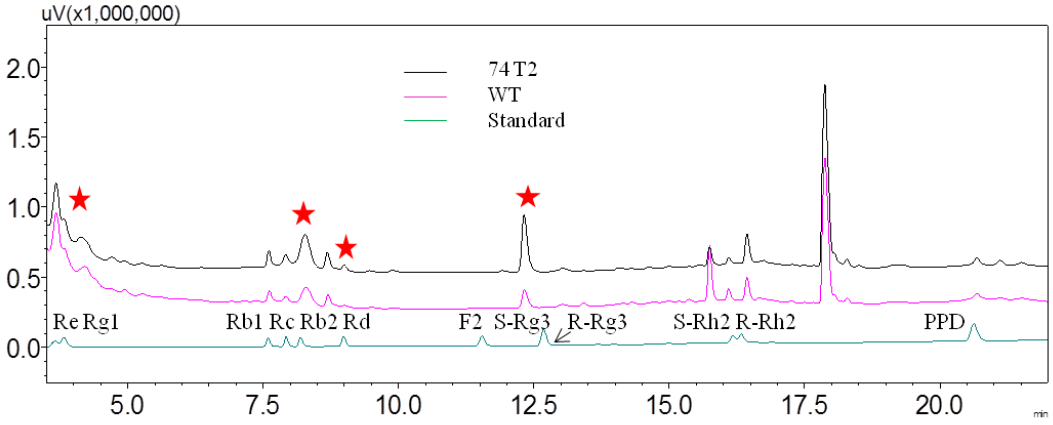
Figure 11. Effects of plant hormones on ginsenoside content in AR and CR roots. Data are shown as means ± standard deviation of values obtained from three experiments.

Table 8. Ginsenoside concentrations of AR and CR roots cultured on NI and IBA medium.

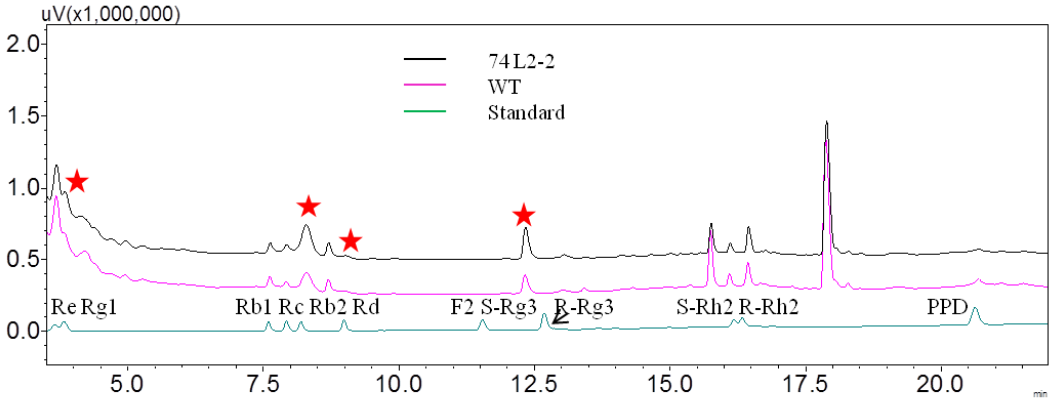
Lines	Ginsenoside concentration (mg/g dry weight)												Total
	PPT type		PPD type										
	Re	Rg1	Rb1	Rc	Rb2	Rd	F2	S-Rg3	R-Rg3	S-Rh2	R-Rh2	PPD	
ARIBA	0.84±0.26	0.3±0.018	0.71±0.026	0.37±0.027	2.83±0.27	0.09±0.01	ND	ND	ND	0.2±0.003	ND	0.02±0.001	5.36±0.92
CRIBA	1.2±0.044	0.21±0.01	0.57±0.043	0.36±0.007	5.07±0.46	0.13±0.006	ND	ND	ND	0.16±0.002	ND	0.02±0.002	7.72±1.7
ARNI	0.53±0.073	0.22±0.019	0.24±0.013	0.13±0.013	1.98±0.15	0.04±0.003	ND	ND	ND	0.13±0.003	ND	0.1±0.005	3.37±0.65
CRNI	0.47±0.012	0.19±0.015	0.25±0.015	0.13±0.006	1.68±0.15	0.04±0.002	ND	ND	ND	0.02±0.006	ND	0.12±0.006	2.9±0.55

The results represent the means±standard deviation obtained from three repeats.

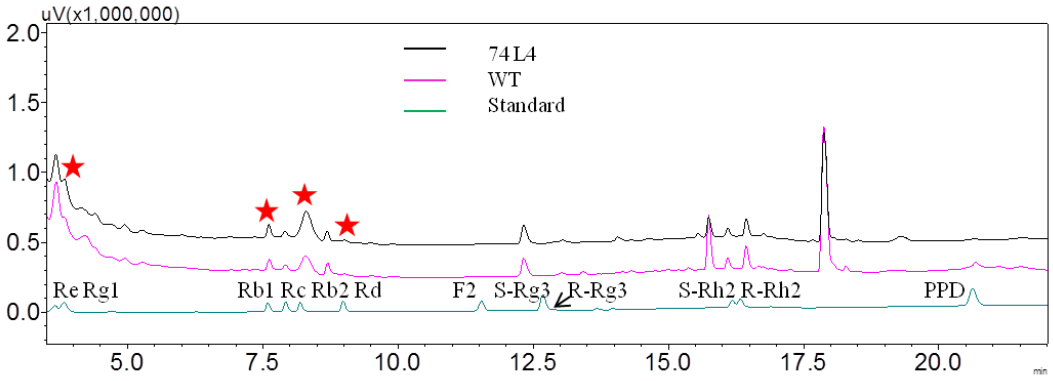
74 T2



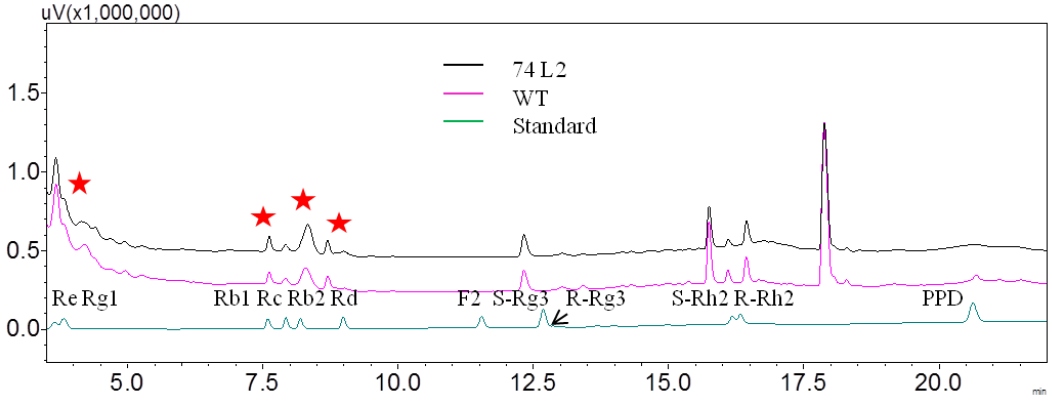
74 L2-2



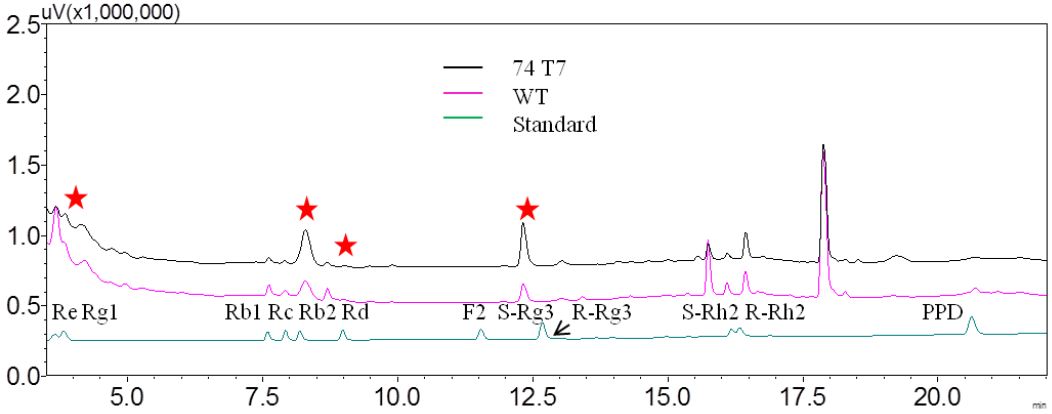
74 L4



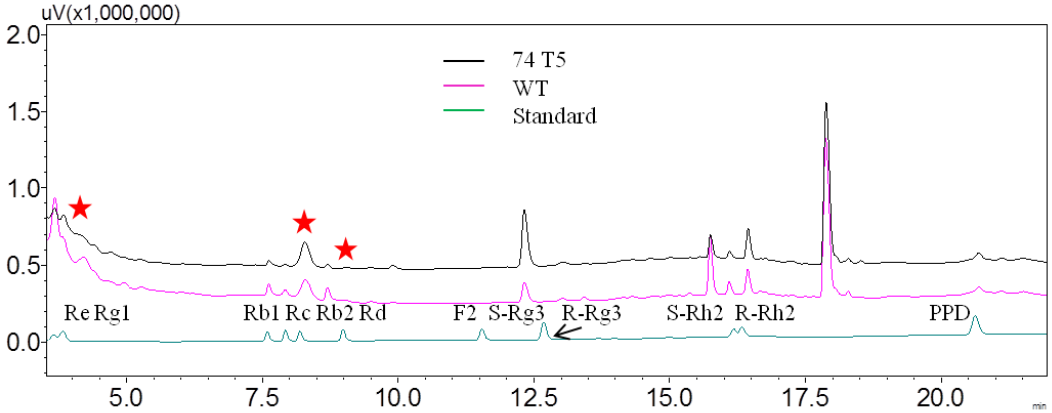
74 L2



74 T7



74 T5



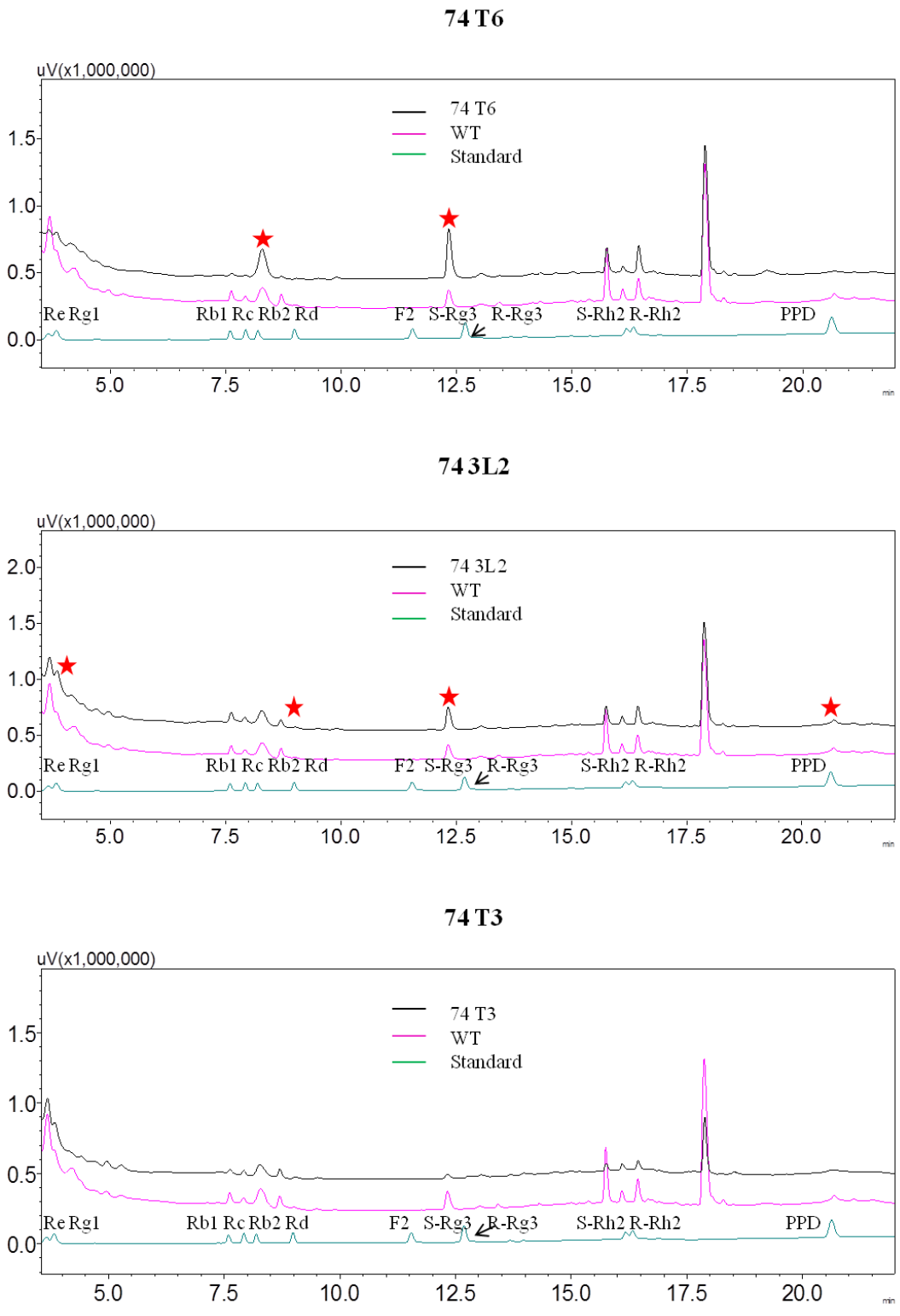


Figure 12. Analysis of ginsenoside contents of PgUGT74AE2 transgenic lines by HPLC.

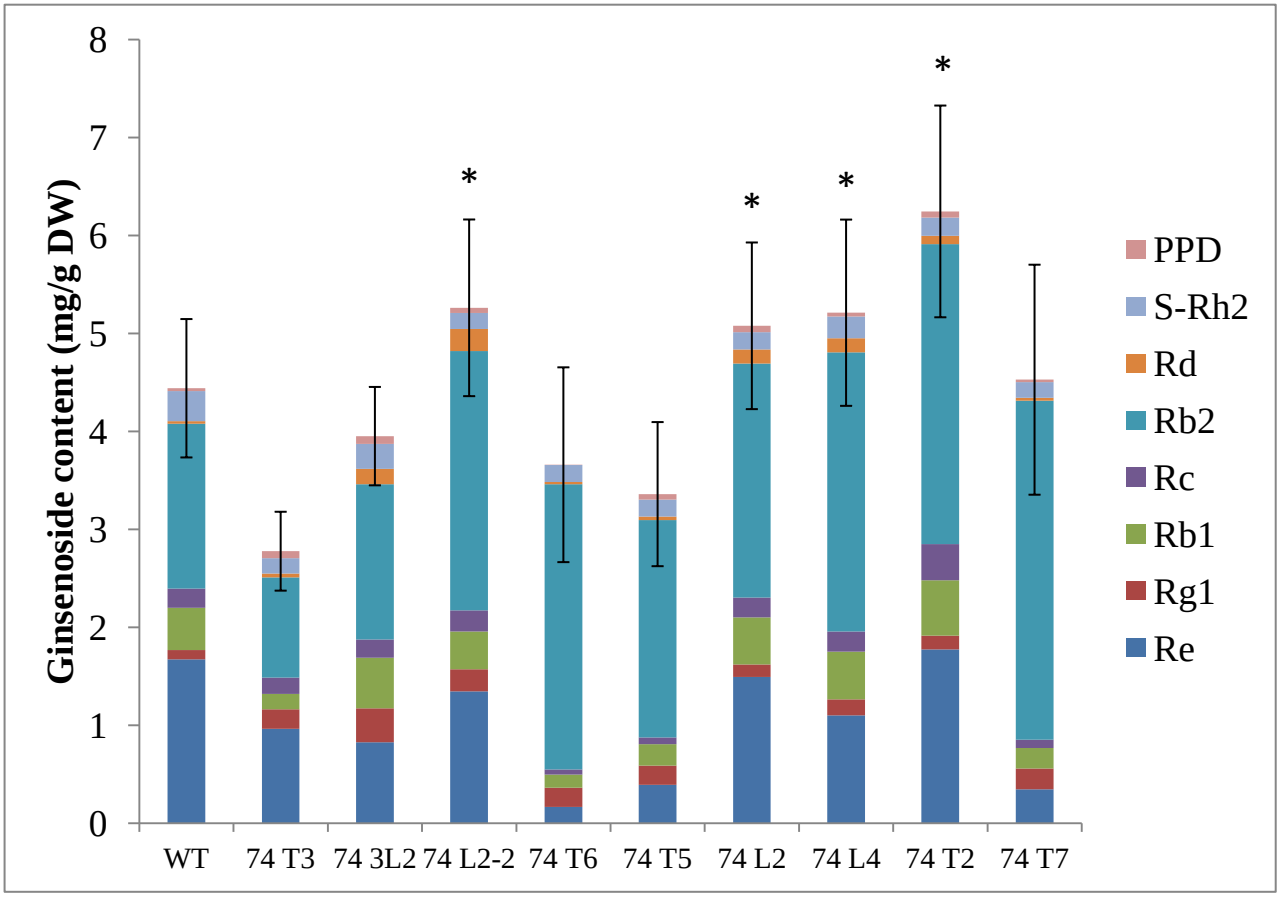


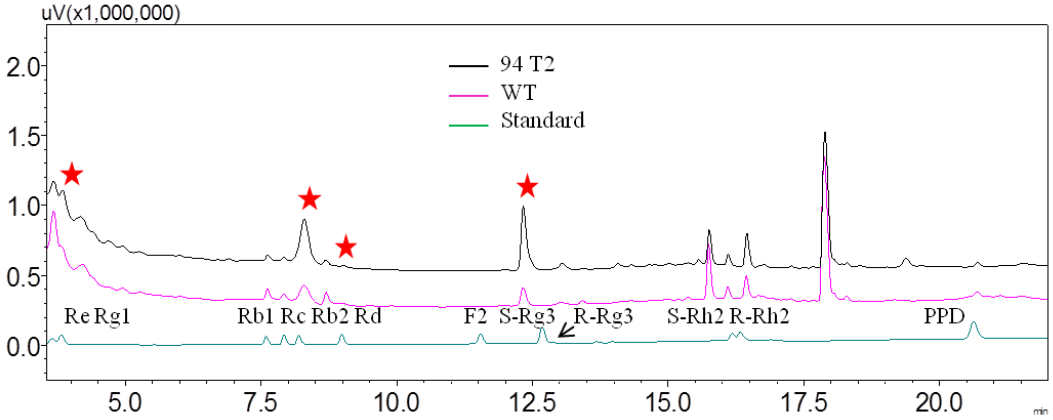
Figure 13. Ginsenosides concentration of PgUGT74AE2 transgenic lines.

Table 9. Ginsenosides concentration of PgUGT74AE2 transgenic lines.

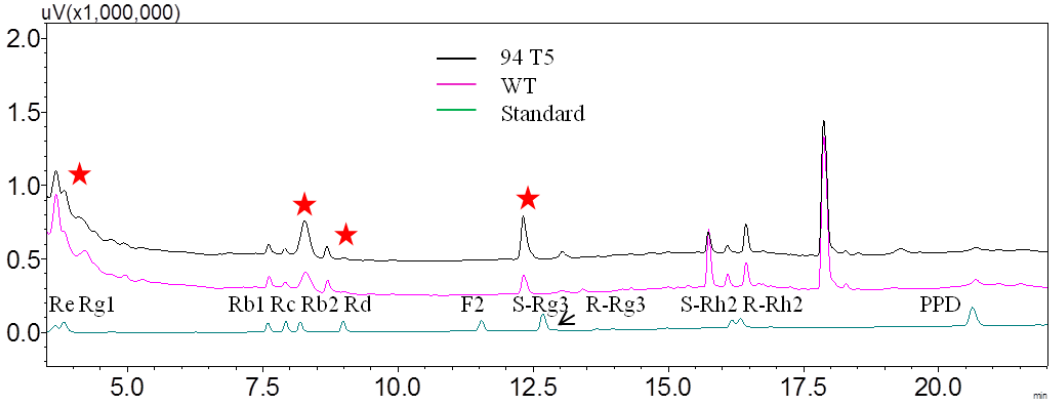
Lines	Ginsenoside concentration (mg/g dry weight)												Total
	PPT type		PPD type										
	Re	Rg1	Rb1	Rc	Rb2	Rd	F2	S-Rg3	R-Rg3	S-Rh2	R-Rh2	PPD	
WT	1.67±0.023	0.097±0.013	0.44±0.02	0.20±0.003	1.68±0.05	0.025±0.002	ND	ND	ND	0.31±0.006	ND	0.03±0.004	4.44±0.7
74 T3	0.96±0.023	0.20±0.024	0.16±0.01	0.17±0.01	1.02±0.07	0.031±0.001	ND	ND	ND	0.16±0.004	ND	0.07±0.03	2.78±0.4
74 3L2	0.83±0.18	0.35±0.099	0.51±0.015	0.19±0.005	1.59±0.06	0.16±0.008	ND	ND	ND	0.25±0.004	ND	0.078±0.02	3.95±0.5
74 L2-2	1.35±0.074	0.23±0.014	0.39±0.008	0.22±0.023	2.65±0.09	0.23±0.007	ND	ND	ND	0.16±0.001	ND	0.052±0.026	5.26±0.9
74 T6	0.17±0.011	0.20±0.031	0.13±0.01	0.051±0.002	2.91±0.07	0.023±0.005	ND	ND	ND	0.17±0.002	ND	0.005±0.003	3.66±0.99
74 T5	0.39±0.035	0.19±0.04	0.22±0.016	0.069±0.009	2.22±0.04	0.037±0.005	ND	ND	ND	0.17±0.001	ND	0.057±0.012	3.36±0.74
74 L2	1.49±0.072	0.13±0.025	0.48±0.024	0.20±0.033	2.39±0.08	0.14±0.008	ND	ND	ND	0.18±0.01	ND	0.066±0.02	5.08±0.85
74 L4	1.10±0.036	0.16±0.046	0.49±0.003	0.20±0.01	2.85±0.1	0.14±0.008	ND	ND	ND	0.22±0.006	ND	0.040±0.01	5.21±0.95
74 T2	1.78±0.072	0.14±0.033	0.57±0.01	0.37±0.021	3.06±0.1	0.083±0.002	ND	ND	ND	0.19±0.014	ND	0.064±0.001	6.25±1.0
74 T7	0.35±0.024	0.21±0.01	0.21±0.008	0.086±0.002	3.46±0.03	0.030±0.003	ND	ND	ND	0.16±0.002	ND	0.023±0.012	4.53±1.2

The results represent the means ±standard deviation obtained from three repeats.

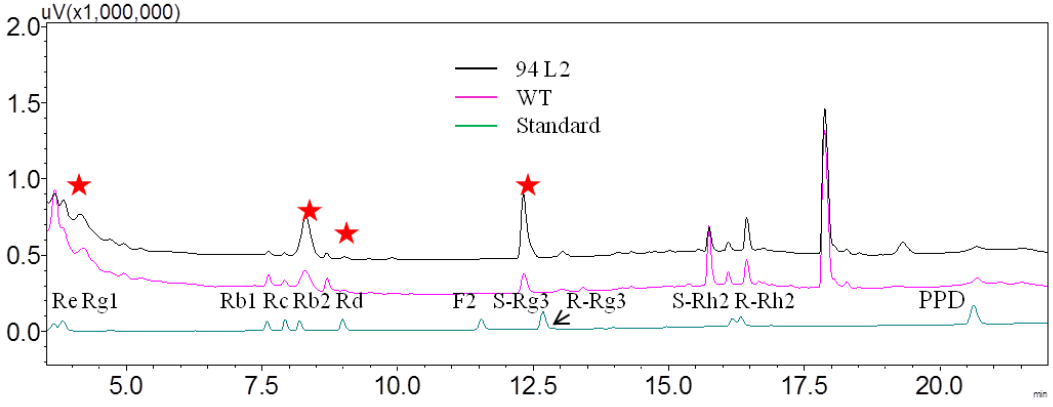
94 T2



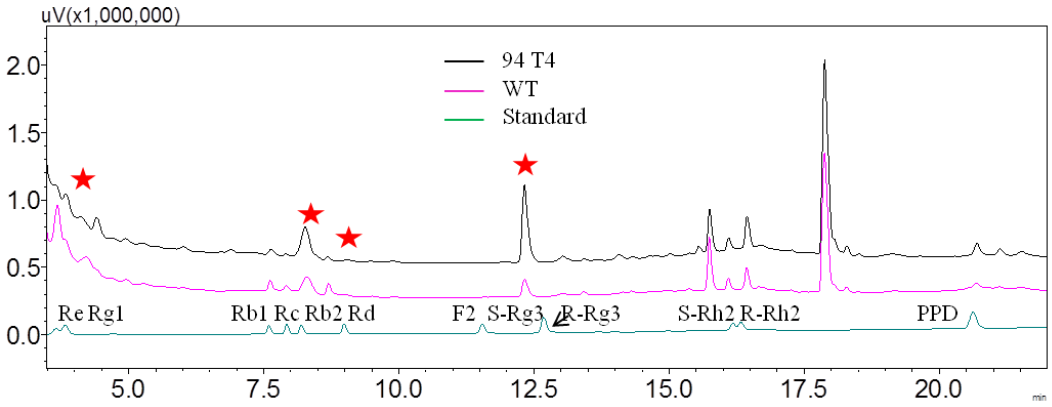
94 T5



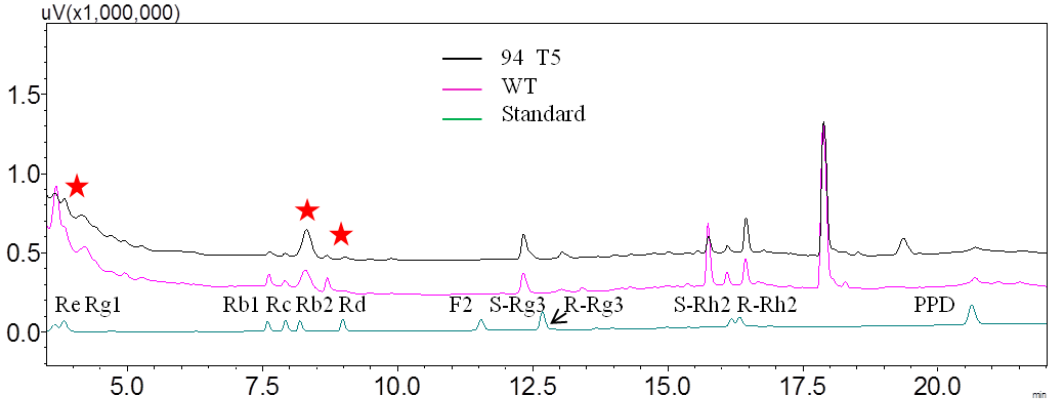
94 L2



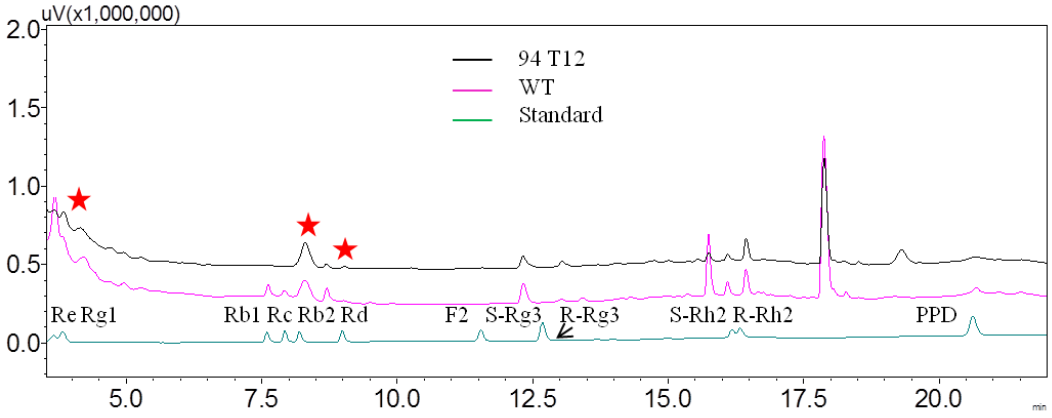
94 T4



94 T8



94 T12



94 T11

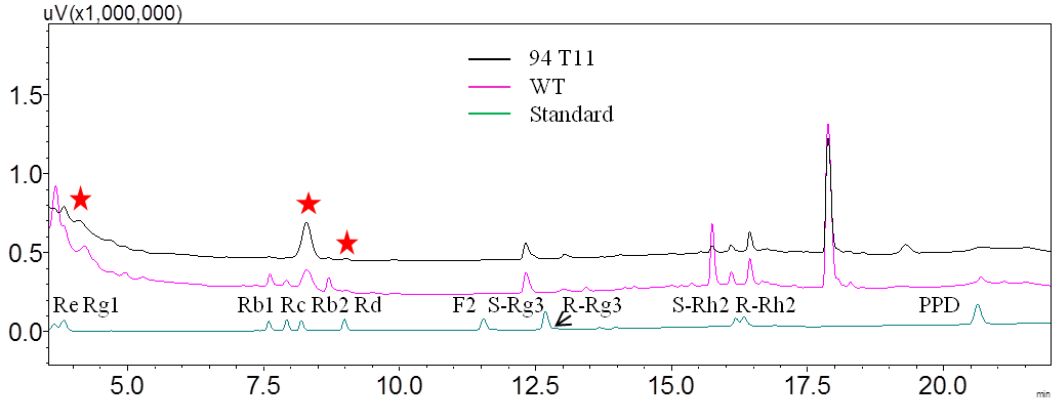


Figure 14. Analysis of ginsenoside contents of PgUGT94Q2 transgenic lines by HPLC.

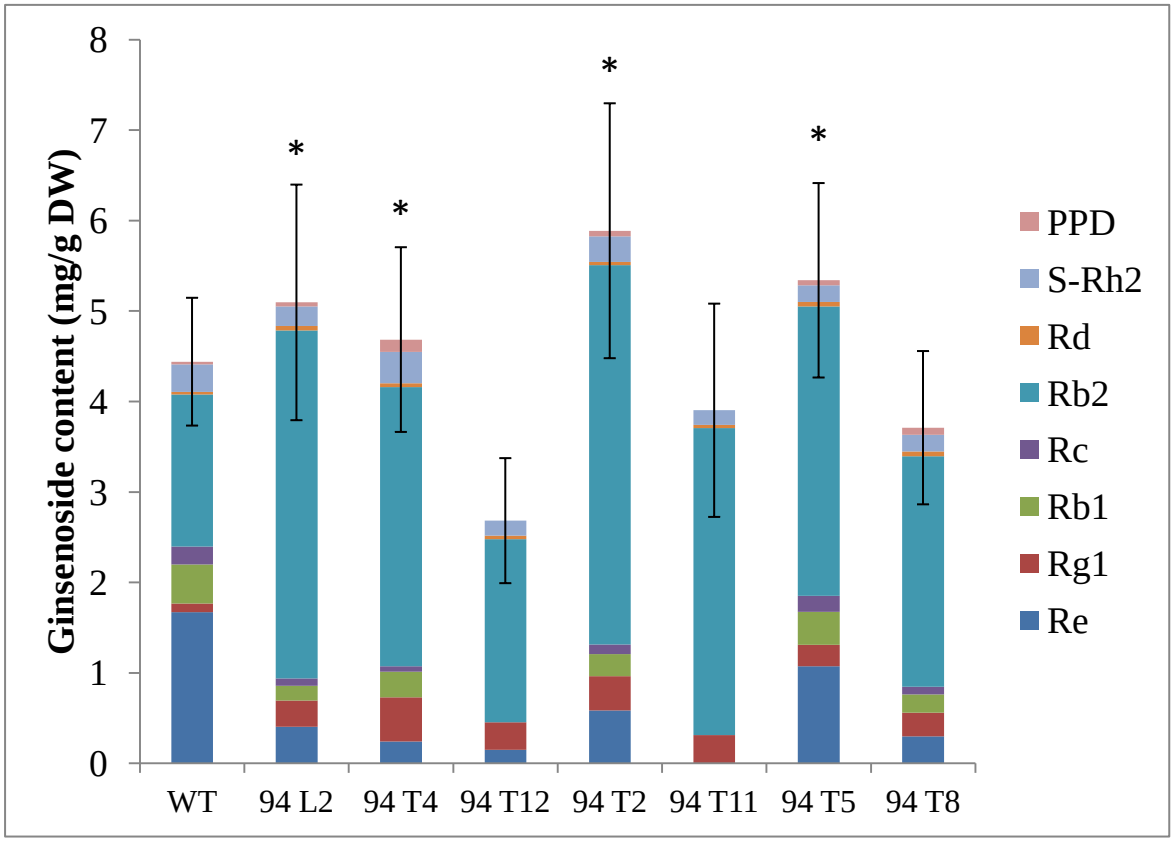


Figure 15. Ginsenosides concentration of PgUGT94Q2 transgenic lines.

Table 10. Ginsenosides concentration of PgUGT94Q2 transgenic lines.

Lines	Ginsenoside concentration (mg/g dry weight)											Total
	PPT type		PPD type									
	Re	Rg1	Rb1	Rc	Rb2	Rd	F2	S-Rg3	R-Rg3	S-Rh2	PPD	
WT	1.67±0.02	0.097±0.01	0.44±0.02	0.20±0.003	1.68±0.05	0.025±0.002	ND	ND	ND	0.31±0.006	0.03±0.004	4.44±0.7
94 L2	0.40±0.02	0.29±0.03	0.16±0.001	0.084±0.01	3.84±0.04	0.049±0.003	ND	ND	ND	0.22±0.008	0.043±0.004	5.10±0.7
94 T4	0.24±0.01	0.49±0.11	0.29±0.03	0.058±0.008	3.09±0.04	0.045±0.004	ND	ND	ND	0.35±0.006	0.13±0.009	4.68±0.9
94 T12	0.15±0.09	0.30±0.01	ND	ND	2.02±0.09	0.04±0.007	ND	ND	ND	0.17±0.002	ND	2.68±0.69
94 T2	0.59±0.02	0.38±0.05	0.24±0.001	0.11±0.02	4.19±0.06	0.04±0.006	ND	ND	ND	0.28±0.003	0.06±0.02	5.89±0.62
94 T11	ND	0.31±0.009	ND	ND	3.39±0.03	0.036±0.003	ND	ND	ND	0.16±0.005	ND	3.90±0.84
94 T5	1.07±0.03	0.24±0.04	0.36±0.03	0.18±0.006	3.2±0.04	0.05±0.003	ND	ND	ND	0.18±0.003	0.06±0.007	5.34±0.85
94 T8	0.3±0.01	0.26±0.05	0.20±0.01	0.085±0.005	2.55±0.1	0.054±0.009	ND	ND	ND	0.18±0.005	0.079±0.008	3.71±0.85

The results represent the means ±standard deviation obtained from three repeats.

DISCUSSION

Over-expression of endogenous UDP-glycosyltransferases genes in Korean wild ginseng results in a reasonable improvement of ginsenoside Rb2 and Rd, but Rg1 glycosylated by unknown UDP-glycosyltransferases also increased concentration. Transgenic lines with relative high expression (PgUGT74AE2 T5, 94 T4, 94 L2, 94 T2; PgUGT74AE2 L2-2, 74 L2, 74 L4, 74 T2) showed content enhancement of one unknown compound, that need to be confirmed in the future (LC-MS is on the progress to analyze unknown compound).

PgUGT74AE2 and PgUGT74AE2 encoded UDP-glycosyltransferases reported in yeast system that corresponding to Rh2 and Rg3 synthesis, PgUGT74AE2 add 1 glucose at C3 and PgUGT94Q2 add 1 glucose at glucose side chain of C3, PgUGT71A27 add 1 glucose at C20. PgUGT74AE2 and PgUGT94Q2 worked same function in synthesis of F2 and Rd (Jung et al. 2014) (Figure 16). But over-expression of these two UGTs in wild-type ginseng didn't show contents improvement of Rh2 and Rg3. In the protopanaxtriol (PPT) type ginsenosides biosynthesis pathway, UGTPg 101 performed multiple functions that could add 1 glucose at both C20 and C6 site, while UGTPg100 is not specific of substrate that could add 1 glucose at C6 site of ginsenosides F1 and PPT (Wei et al. 2015) (Figure 17). Based on the reported researches, we could Figureure out that UDP-glycosyltransferases work very complicatedly involved of ginsenosides biosynthesis pathway inside of ginseng plants. As Rh2 and Rg3 are the specific ginsenosides which have been reported existence in red and processed ginseng, PgUGT74AE2 and PgUGT94Q2 encoded UDP-glycosyltransferases corresponding biosynthesis pathway inside of natural ginseng plants deserved to be verified.

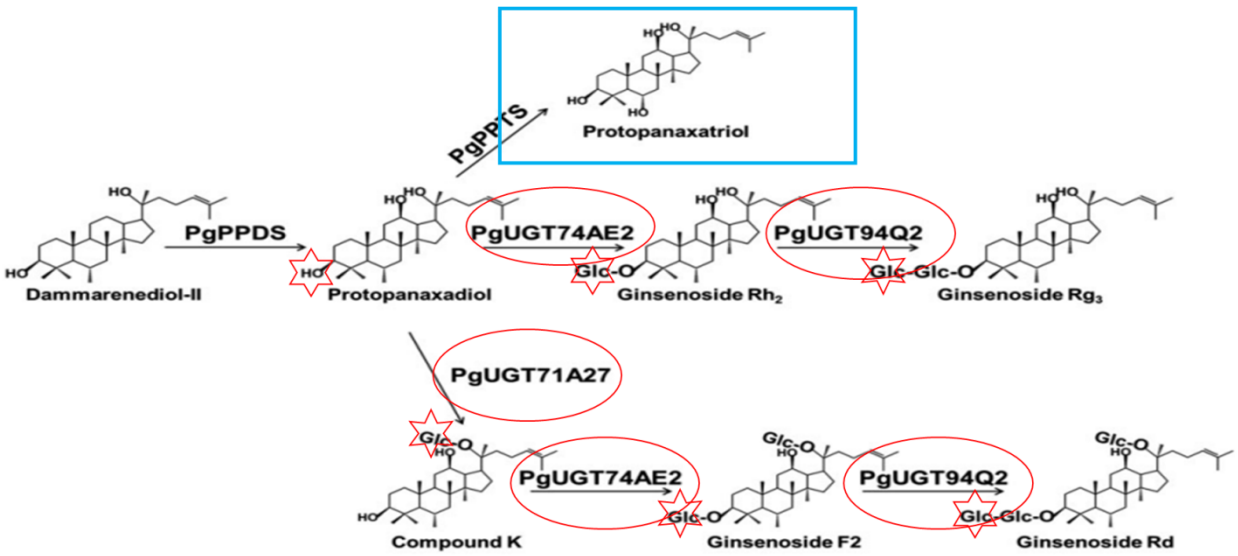


Figure 16. PgUGT74AE2 and PgUGT94Q2 involved ginsenosides biosynthesis pathway demonstrated in yeast system (Jung et al. 2014).

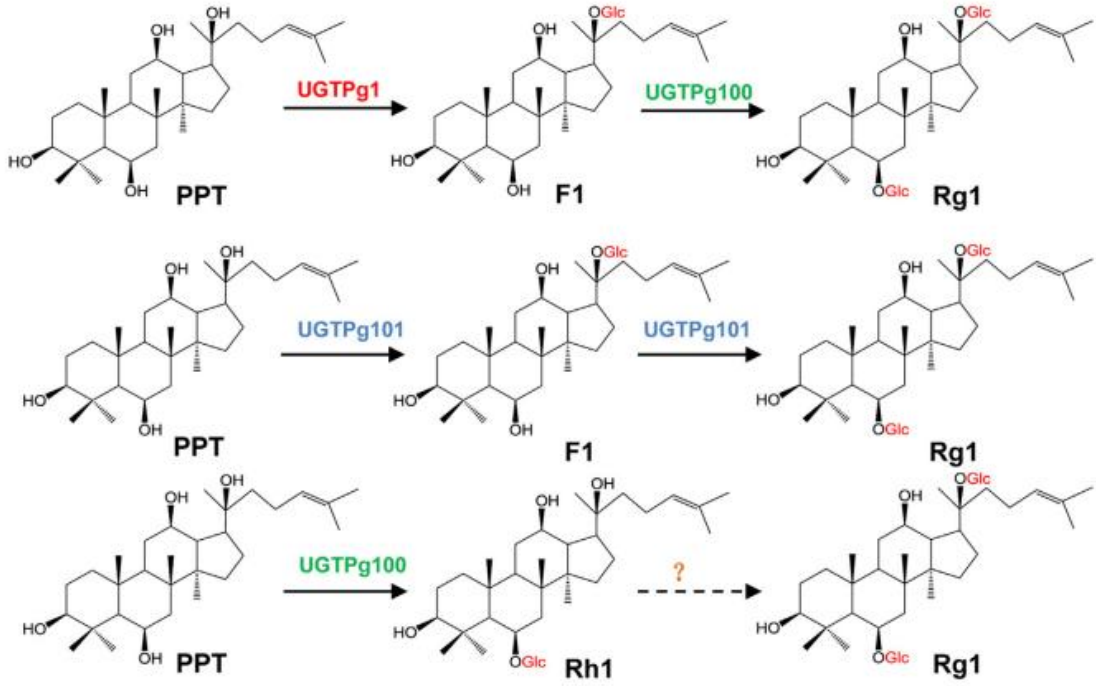


Figure 17. UDP-glycosyltransferases genes involved in ginsenosides biosynthesis pathway (Wei et al. 2015).

CONCLUSION

Adventitious roots induction procedure of ginseng has been optimized, callus-derived adventitious roots induced by IBA performed enhancement of total 12 selected ginsenoside. Ginseng transgenic lines could be screened from transgenic candidate adventitious roots which induced from transfected callus directly.

Over-expression of endogenous UDP-glycosyltransferases genes in Korean wild ginseng couldn’t increase the contents of ginsenoside Rg3 and Rh2 directly, but enhanced the contents of possible down-stream ginsenoside Rb2, Rd and Rg1. Rb2 and Rd have been reported lied on the down-stream synthesis pathway behind Rh2 and glycosylated by unknown UGTs. And Rg1 lied on the branch pathway divided from PPD, then glycosylated by other unknown UGTs.

Through over-expression of PgUGT74AE2 and PgUGT94Q2 respectively in Korean wild ginseng, we produced transgenic ginseng lines with enrichment in Rg1, Rb2 and Rd. Ginsenoside Rg1 has the effcacy of improving cerebral and liver functions, adjusting blood pressure and anti-fatigue and anti-stress activities. Ginsenoside Rd has efficacy of relieving pain. Ginsenoside Rb2 has efficacy of relieving pain, inhibiting the metastasis of cancer cells and anti-diabetic efficacy (Choi et al. 2008)

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