A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Improving ginsenoside content in Korean wild ginseng (*Panax ginseng* Meyer) through different biotechnological approaches

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A thesis submitted in partial fulfillment of the requirement for the degree of
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요약문

인삼은 동양사람들에게 널리 이용되어온 가장 오래 된 약초 중의 하나이고, 이 인삼의 주요 성분인 진세노사이드는 30 여종이 알려져 있다. 감마선(γ-ray)이 산삼 부정근의 성장 및 진세노사이드 함량에 미치는 영향을 평가하기 위해, 산삼 뿌리 유래의 부정근에 25, 50, 75, 100, 125Gy 의 감마선을 조사하여 돌연변이를 유도하였다. 감마선(^{60}Co) 조사는 제주대학교 원자력과학기술연구소에서 수행하였다. 감마선을 조사한 부정근은 30 일간 플라스크 배양한 후, 형성된 secondary root 의 수를 조사하여 선발하였다. 감마선을 조사하지 않은 야생형과 선발된 변이 부정근의 진세노사이드 (Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 와 Rd) 함량은 HPLC 와 PDA 검출기를 이용하여 측정하였으며, Digoxin 을 internal standard 로 사용하였다. 그 결과, 50Gy 이상의 방사선을 조사한 처리구에서는 secondary root 의 수가 야생형에 비해 적었고, 50Gy 이하의 방사선을 조사한 처리구에서 야생형보다 secondary root 의 수가 많은 변이 부정근을 선발할 수 있었다. 따라서 진세노사이드의 생산성을 높이기 위한 방사선량으로는 50Gy 이하가 적합함을 알 수 있었다. Secondary root 의 수가 야생형보다 많은 5 개의 계통을 선발한 후, 한달 간 플라스크에 배양하여 건조중량을 측정한 결과, 야생형은 0.31g 이었고, 선발한 1, 3, 9, 13 및 14 번 계통들은 각각 0.46g, 0.45g, 0.29g, 0.34g, 0.48g 이었다. 생장율은 야생형이 4.42 였으며, 선발한 계통들인 1, 3, 9, 13, 14 번은 각각 6.57, 6.48, 4.10, 4.90, 6.90 이었다. 선발한 1, 3, 14 번 계통들의 생장율은 야생형에 비해 각각 49%, 47%, 57% 증가하였다.

총 진세노사이드 함량은 1 번 계통에서 가장 크게 증가하였고, 1 번 계통의 총 진세노사이드 (Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2, Rd) 함량은 야생형과 비교하여 약 1.6 배 증가하였다. 또한, 이 계통의 진세노사이드 중 Rg1, Re, Rh1 과 Rb2 의 함량은 야생형에 비해 2-3 배 증가하였음을 알 수 있었다. 3 번 계통의 진세노사이드 Re, Rh1, Rb2, Rc 와 Rd 함량은 야생형에 비해 1.6-2.3 배 증가하였으며, 9 번 계통에서는 진세노사이드 Rg1, Re 와 Rh1 함량이 야생형에 비해 1.7-2 배 증가하였다. 4 번 계통의 진세노사이드 함량은 야생형과 비슷하였고, 13 번 계통의 진세노사이드 함량은 야생형에 비해 감소하였음을 알 수 있었다. 이들 결과로부터, 선발한 변이 계통들 중 1 번 계통이 가장 높은 진세노사이드 함량을 가지고 있음을 알 수 있었다.

본 연구결과 우리는 biomass 생산성과 진세노사이드 함량이 크게 증가된 돌연변이 세포주를 선발할 수 있었으며, 감마선 조사는 2 차 대사산물 특히 진세노사이드의 생산량 증대에 있어 강력하고 유용한 수단임을 확인할 수 있었다. 이 결과는 감마선 조사가 진세노사이드 합성에 필요한 효소의 활성 등에 영향을 미쳤음을 시사한다.

인삼은 성장이 느리고 긴 생산주기 (4-6 년)를 가지고 있는 다년생 식물이며, 종자를 생산하기까지 3 년이상의 유년기가 필요하다. 이러한 특성 때문에 전통육종에 의한 우수 유전자형의 창출은 매우 어려운 실정이다. 또한, 지금까지 산삼의 부정근에 방사선을 조사한 후 그 돌연변이 부정근을 재분화한 보고는 없다. 따라서, 본 연구에서는 인삼의 신품종 육성을 위해 진세노사이드 함량이 증가된 변이 세포주를 이용한 식물 재분화계를 확립하였다. 여기서 확립된

재분화 시스템은 인삼식물의 형질전환에도 활용될 수 있을 것이다.

한국 산삼의 체세포배발생과 식물체 재분화를 위하여 부정근 유래의 캘러스로부터 체세포배를 유도한 결과, 0.5 mg/L 2,4-D 가 포함된 배지에서 가장 높은 빈도로 유도되었고, 야생형과 돌연변이 부정근의 체세포배 형성 빈도는 각각 15.3% 와 14.7% 였다. 야생형과 돌연변이 부정근의 체세포배 형성수는 각 캘러스 당 각각 25.6 개와 23.7 개였으며, 야생형과 돌연변이 부정근의 체세포배 형성 빈도에는 유의 차가 없었다. Globular 형태의 체세포배는 배발생 캘러스의 표면에 형성되었다.

체세포배발생으로부터 식물체 재분화에 미치는 GA_3 의 영향을 평가한 결과, GA_3 무처리구에서는 36%의 재분화효율을 보였으나, 5 mg/L 의 GA_3 처리구에서는 85% 로 가장 높은 재분화효율을 보였다. 배의 성숙과 재분화는 GA_3 의 농도에 크게 영향을 받았으며, 발아한 배는 5 mg/L GA_3가 포함된 MS 배지에서 식물체로 발달하여 성장하였다.

뿌리가 형성되지 않은 식물체는 MS 또는 SH 기본 배지에 0.25 mg/L NAA 혹은 0.5% 활성탄이 포함된 배지에 옮겨 약 1 달후 뿌리유도 효율을 평가하였다. 그 결과, 유도된 뿌리의 질은, 0.25 mg/L NAA 와 1%의 sucurose 가 첨가된 1/3 SH 배지에서 빠르고 두껍게 성장하여 가장 좋은 결과를 보였다. 0.5%의 활성탄과 2% sucrose 가 첨가된 1/3 SH 배지에서 뿌리 유도 효율이 가장 높았으나, 이 배지에서 유도된 뿌리는 잘 자라는 반면 약한 단점이 있었다. 따라서 뿌리유도를 위한 최적 배지는 0.25 mg/L NAA 와 1%의 설탕을 첨가한 1/3 SH 배지였다.

뿌리가 잘 발달한 산삼 재분화 식물체는 인공 혼합토 [peatmoss:vermiculite:

perlite=2:3:1(v/v)]를 넣은 플라스틱 화분 (10x18cm)에 이식하여 생장실에서 순화하였다. 순화 3 개월 후 신초가 형성되어 성장하기 시작하였으며 생존율은 약 30% 였다.

본 연구에서는 중요한 약용식물인 산삼의 효율적인 재분화계를 확립하였고, 이 프로토콜은 한국 산삼의 빠른 대량 생산을 가능하게 해줄 것으로 기대된다. 산삼 부정근으로부터 캘러스를 유도하여 재분화 식물을 얻기까지는 약 6-8 개월이 소요되었다. 본 연구에서는 방사선조사를 통해 얻은 돌연변이 부정근으로부터 식물체를 재분화하였고, 감마선을 이용한 돌연변이 기술과 조직배양에 의한 식물재분화 기술의 융합은 인삼 품종개량에 있어서 효과적인 방법의 하나가 될 수 있을 것으로 생각된다.

산삼 돌연변이 부정근의 진세노사이드 생산성을 개량하기 위하여 합성 스쿠알렌 신타아제(*PgSS2*) 유전자를 35S 프로모터하에 도입한 후 산삼의 돌연변이 부정근에 형질전환을 수행하였다. 형질전환은 돌연변이 산삼 부정근 계통에서 선발한 배발생캘러스를 *PgSS2* 유전자와 *bar* 유전자를 포함하는 아그로박테리움 EHA105 에 감염하여 수행하였다. 형질전환체는 phosphinothricin (PPT) 3mg/L 를 첨가한 MS 배지에서 선발하였다. 그 결과, 10 개체의 PPT-저항성 식물을 선발하였고, 이 식물체들의 유전자 도입은 PAT strip test 와 Southern blot 분석을 통하여 확인하였다. 유전자의 도입이 확인된 산삼 형질전환 식물을 이용하여 스쿠알렌 신타아제(*PgSS2*) 유전자가 진세노사이드의 생합성에서 어떤 역할을 하는지를 조사하였다. 형질전환 산삼 식물 계통들에서 재분화시킨 부정근을 한달 간 배양한 후 총 진세노사이드 함량을 분석한 결과, 형질전환

계통 1, 2, 5, 7, 8 번은 비형질전환 돌연변이 부정근보다 진세노사이드 함량이 약 1.3-1.6 배 증가하였음을 확인하였다. 또한, 이들 형질전환 계통들 중 1 번과 8 번의 진세노사이드 함량이 야생형 및 공벡터가 도입된 계통들보다 약 1.6 배로 가장 높게 나타났다. 이들 결과로부터 형질전환 기술은 진세노사이드 축적이라는 관점에서 인삼의 개량을 용이하게 해 줄 것으로 생각된다.

감마선을 이용한 돌연변이 기술, 식물재분화 및 형질전환 기술의 융합은 새로운 인삼 품종의 개발에 매우 효과적인 방법의 하나가 될 것이다.

ABBREVIATIONS

NAA	Naphthalene acetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
GA	Giberellic acid
HPLC-PDA	High performance liquid chromatography-photodiode array detector
ACN	Acetonitrile
MS	Murashige & Skoog
FW	Fresh weight
DW	Dry weight
PD	Panaxadiol
PT	Panaxatriol
MJ	Methyl jasmonate
UV	Ultraviolet
Gy	Gray
Bar	Phosphinothricin acetyltransferase
PPT	Phosphinothricin
MCL1	Mutant control line
CL	Cell line
SS	Squalene synthase

LB	Left border
RB	Right border
p35S	CaMV 35S promoter
RT-PCR	Reverse transcriptase polymerase chain reaction
2,4-D	2,4-dichlorophenoxy-acetic acid
DEPC	Diethylpyrocarbonate
SEM	Standard error of mean
SH	Schenk & Hidebrandt Medium
LOD	Limit of detection
LOQ	Limit of quantification
LD	Lethal dose
SPSS	Statistical Package for the Social Sciences
S/N	Signal-to-noise

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were extracted from adventitious roots cultured in the flask.

Figure 37. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-4. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 38. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-5. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 39. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-6. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 40. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-7. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 41. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-8. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 42. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-9. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 43. Total ginsenoside content of the mutant control (MCL1) and transgenic lines of *Panax ginseng*.

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SUMMARY

Panax ginseng (Korean wild ginseng) is one of the oldest and the most widely used herbal medicines for the oriental people. Ginsenosides are the major active components in the ginseng and more than 30 ginsenosides were identified from the plant. In order to evaluate effects of γ-irradiation on adventitious root growth and ginsenoside content, adventitious roots of *Panax ginseng* were treated with γ-ray of 25, 50, 75, 100 and 125Gy to induce mutation. The γ-irradiation was performed from ^{60}Co source at the Applied Radiological Science Research Institute, Jeju National University. Cell lines irradiated by γ-ray were cultured in flask for 30 days and selected based on the secondary root numbers of the irradiated adventitious roots. The contents of ginsenosides Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd in wild type and the selected cell lines were evaluated by HPLC-PDA detector with digoxin as the internal standard. After irradiation, below 50Gy, secondary root number of irradiated adventitious root was more than that of non-irradiated adventitious root. Otherwise, over 50Gy, the secondary root number was less than that of non-irradiated control. Therefore, the dosage below 50Gy was considered as adequate for the induction of mutant cell lines of *Panax ginseng*. Five cell lines, CL1, 3, 9, 13, and 14, were selected according to the secondary root number. After 30 days culture in flask, dry weight of control was 0.31g and the selected cell lines CL1, 3, 9, 13 and 14 were 0.46g, 0.45g, 0.29g, 0.34 and 0.48g respectively. The value of growth ratio of control was 4.42 and the selected cell lines CL1, 3, 9, 13, 14 were 6.57, 6.48, 4.1, 4.9 and 6.9, respectively. The values of growth ratio in cell

lines CL1, CL3 and CL14 were increased 49%, 47% and 57%, respectively, compared to the control.

The contents of the eight ginsenosides were significantly enriched, especially in the cell line CL1. The content of total ginsenosides (Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd) in the cell line CL1 was 1.6-fold higher than that of the control. In the cell line CL1, the level of ginsenoside Rg1, Re, Rh1and Rb2 contents were increased by 2-3 times than that of the control. In the cell line CL3, level of ginsenoside Re, Rh1, Rb2, Rc and Rd contents were increased by 1.6-2.3 times than that of the control. In the cell line CL9, level of ginsenoside Rg1, Re and Rh1 contents were increased by 1.7-2 times than that of the control. In the cell line CL14, level of ginsenoside contents were similar with that of the control. In case of the cell line CL13, the ginsenoside contents were affected negatively in ginseng adventitious root cultures. Overall, the highest ginsenoside contents were obtained in cell line CL1.

In this study, I selected cell lines with a significantly increased biomass productivity and ginsenoside content. Based on the results obtained in this report, gamma irradiation is powerful and useful tool for the enhancement of production of secondary metabolites, especially for ginsenoside. This result suggests that gamma irradiation might have triggered the enzyme activities for the synthesis of ginsenosides.

Ginseng is a perennial plant that grows slowly and has a long production cycle (4-6 years). And juvenile period is required longer than three years for producing seeds. Thus, it is very difficult to make superior genotypes by conventional breeding. So, I used the mutant cell lines to establish the best conditions for regeneration system, and also to develop the

new cultivar of *Panax ginseng*. At present no information is available on the regeneration of mutant adventitious root line that has been selected from γ-irradiated *P. ginseng* adventitious roots. I report on an efficient procedure for the regeneration of wild-type and mutant cell lines of *P. ginseng* adventitious roots through somatic embryogenesis. This regeneration system was also used for genetic transformation.

I established an efficient in vitro protocol for somatic embryogenesis and plantlet conversion of Korean wild ginseng (*Panax ginseng* Meyer).The highest induction frequency of somatic embryos was observed on the medium supplemented with 0.5 mg/L 2, 4-D. The frequency of somatic embryo formation in wild-type and the mutant cell line was15.3% and 14.7%, respectively. The number of somatic embryos per callus was 25.6 and 23.7 in wild-type and the mutant cell line, respectively. There was no significant difference in somatic embryo formation frequency between wild-type and the mutant cell line. Globular shaped somatic embryos formed on the surfaces of embryogenic callus.

The optimal concentration of GA_3 in germination medium was 5 mg/L, yielding the highest germination frequency of 85%. Without GA_3 treatment, the germination frequency was lowest at 36%. Maturation and germination of embryos were strongly influenced by the GA_3 concentration. The germinated embryos were developed to shoots and elongated on MS medium with 5 mg/L GA_3.

The shoots without roots were excised and transferred to different rooting media, half or one-third strength MS, or SH basal medium supplemented with 0.25 mg/L NAA or with 0.5% activated charcoal. Adventitious roots formed from the excised regions of the shoots.

After 1 month, the rate of root formation from the shoots was examined. As far as root quality is concerned, 1/3 SH medium with 0.25 mg/L NAA and 1% sucrose showed the best result among the tested rooting media; the roots grew fast and thickened on the medium. Although 1/3 SH medium with 2% sucrose and 0.5% activated charcoal was most effective in inducing roots, the roots grew well but weak. The optimal medium for rooting is therefore 1/3 SH medium supplemented with 0.25 mg/L NAA and 1% sucrose among the tested rooting media in this study. In my comparative studies, SH medium was more effective than MS medium in root induction and proliferation. So, SH medium, especially 1/3 strength SH medium is suitable for root induction and growth of regenerated ginseng plants.

Well-developed plantlets with both shoots and roots derived from adventitious roots were transferred to plastic pots (10×18cm) containing an artificial soil mixture of peatmoss, vermiculite and perlite (2:3:1 v/v) in a growth room. The survival rate of the plantlets was about 30% after 3 months of culture and new leaf began growing.

I developed an efficient *in vitro* regeneration protocol for an important medicinal plant of *P. ginseng*. The protocol described here will allow a relatively rapid mass production of Korean wild ginseng plants. It takes 6-8 months from the callus induction of adventitious roots to the plantation of plants. In the present study, I also produced the regenerated plants from the mutant adventitious roots which were obtained by γ-irradiation. The combination of mutation technique by γ-irradiation and plant regeneration by tissue cultures may be an effective way to ginseng improvement. The protocol established in this study was used for the genetic transformation of this species.

With the purpose of improving ginsenoside production in *Panax ginseng* (*Panax ginseng* Meyer) mutant adventitious root lines, a synthetic gene encoding squalene synthase (*PgSS2*) was placed under the control of 35S promoter and transferred to *Panax ginseng*. The mutant lines used in this study generated by γ-irradiation (^{60}Co) of ginseng adventitious roots. Transgenic plants were generated by *Agrobacterium*-mediated transformation. Embryogenic callus obtained from ginseng adventitious root lines were transformed by infection with *A. tumefaciens* strain EHA105 containing the *PgSS2* gene and the phosphinothricin acetyltransferase (*bar*) gene as a selectable marker. Transformants were selected on Murashige Skoog medium containing 3mg/L phosphinothricin. Ten phosphinothricin-resistant plant lines were generated on selective medium, and the integration of the transgene in these plants was confirmed by PAT test strip, RT-PCR and Southern hybridization. Southern hybridization analysis suggested the insertion of one or multiple copies of T-DNA into ginseng genome. I am investigating the regulatory role of *Panax ginseng* squalene synthase (*PgSS2*) on the biosynthesis of ginsenoside by using the transgenic ginseng plant lines. The total contents of the 8 ginsenoside types were significantly enriched in the 5 transgenic lines compared to the mutant control (MCL1). The line SS 1 showed a 1.6-fold increase than the MCL1. The line SS 2 showed a 1.5-fold increase than the MCL1, and the line SS 5, SS 7 showed a 1.3-fold increase than the MCL1. The total contents of the 8 ginsenoside types (Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd) obtained from the mutant control (MCL1), empty vector and the SS lines were 6.14 mg g^{-1}, 6.07 mg g^{-1}, 9.82 mg g^{-1}, 9.06 mg g^{-1}, 5.01 mg g^{-1}, 4.37 mg g^{-1}, 8.22 mg g^{-1}, 6.46 mg g^{-1}, 8.05

mg g^{-1}, 9.75 mg g^{-1}, 4.56 mg g^{-1}, respectively. Overall, the highest ginsenoside content was obtained with SS line 1 and 8.

This transformation method may facilitate the improvement of *Panax ginseng* in terms of the accumulation levels of ginsenoside. The combination of mutation technique by γ-irradiation, plant regeneration by tissue cultures and genetic transformation may be an effective way to develop new ginseng cultivars.

Chapter I

Improving ginsenoside content in Korean wild ginseng (*Panax ginseng* Meyer) mutant lines induced by γ-irradiation (^{60}Co) of adventitious roots

INTRODUCTION

There are several medicinal *Panax* species identified from all over the world. From a number of speices, *Panax ginseng* (Korean wild ginseng) is one of the oldest and the most widely used herbal medicines and this species is almost extinct in wild habitats. Fortunately, *Panax ginseng* is widely cultivated in Korea, China, Japan, and several countries in North America and Europe under special shade conditions (Hobbs 1996, Mabberley 1987, Toda *et al.* 2003, Nam *et al.* 2002). Depending on the species, growth condition and location etc, the ginsenoside contents vary widely which act on the central nervous system, cardiovascular system, endocrine system, and immune system. These active ingredients also increase endocrine secretion, promote immune function, and have anti-aging and stress relieving effects (Briskin 2000, Shibata 2001, Vogler *et al.* 1999). *Panax ginseng* (Korean ginseng) and *Panax quinquefolius* (American ginseng) are characterized well by phytochemistry (Mallol *et al.* 2001, Ngan *et al.* 1999) and more than 30 ginsenosides were identified from this genus. They can be classified into three groups based on their aglycones (Figure 1): the protopanaxadiol-type, protopanaxatriol-type, and oleanane type saponins (Jung *et al.* 2003, Nah 1997).

Ginsenoside	R_1	R_2
Rh$_2$	Glc	H
Rg$_3$	Glc^2-Glc	H
Rd	Glc^2-Glc	Glc
Malonyl-Rd	Glc^2-Glc^6-Ma	Glc
Rb$_2$	Glc^2-Glc	Glc^6-Ara$p$
Malonyl-Rb$_2$	Glc^2-Glc^6-Ma	Glc^6-Ara$p$
Rc	Glc^2-Glc	Glc^6-Ara$f$
Malonyl-Rc	Glc^2-Glc^6-Ma	Glc^6-Ara$f$
Rb$_1$	Glc^2-Glc	Glc^6-Glc
Malonyl-Rb$_1$	Glc^2-Glc^6-Ma	Glc^6-Glc
Rb$_3$	Glc^2-Glc	Glc^6-Xyl
Ra$_1$	Glc^2-Glc	Glc^6-Arap^4-Xyl
Ra$_2$	Glc^2-Glc	Glc^6-Araf^2-Xyl
Ra$_3$	Glc^2-Glc	Glc^6-Glc^3-Xyl

Ginsenoside	R_1	R_2	R_3
Rh$_1$	H	H	Glc
Rg$_1$	H	Glc	Glc
Rf	H	H	Glc^2-Glc
20-Glc-Rf	H	Glc	Glc^2-Glc
Rg$_2$	H	H	Glc^2-Rha
Re	H	Glc	Glc^2-Rha

R_1	R_2
Glc^2-Glc	Glc

Glc= β-D-glucose   Rha= α-L-rhamnose
Ara$p$= α-L-arabinose (pyranose)
Ara$f$= α-L-arabinose (furanose)
Xyl= β-D-xylose   Ma= malonyl

Figure 1. Structures of ginsenosides isolated from *Panax ginseng*.

The both species are used for preparation of tonic, prophylactic and anti-ageing agents (Chang and But 1986). Some ginsenosides are used widely, such as Rg1, Re, Rb1 are used to determinate ginseng quality (Wu *et al*. 2007). Some ginsenosides, like Rg3, Rh2 and Rh1 are rarely detected in cultured ginseng. Ginsenoside Rb1, Re, Rg1 and Rb2 were the precursors

to form other ginsenosides by Human Intestinal Microflora and steaming. Different forms of ginsenosides showed several different functions.

Ginsenoside Rg1: Ginsenoside Rg1 inhibits the rat brain cAMP phosphodiesterase activity, excitatory amino acids are involved in neuronal survival, synaptogenesis, neuronal plasticity, learning and memory processes, estrogen-like activity, and promoted functional neovascularization into a polymer scaffold in vivo (Stancheva *et al*. 1993, Balazs *et al*. 1998, Muller *et al*. 1998, Chan *et al*. 2002, Sengupta *et al*. 2004).

Ginsenoside Rb1: Ginsenoside Rb1 successfully improved homocysteine-induced reduction of endothelial nitric oxide synthase expression and reduced homocysteine-induced oxidative stress (Zou *et al*. 2005). Preconditioning of Rb1 showed protective effects on myocardial ischemia and reperfusion injury (Wang *et al*. 2008). Rb1 promoted neurotransmitter release (Xue *et al*. 2006) and used to prevent or treat gastritis and gastric ulcers (Jeong *et al*. 2003).

Ginsenoside Re: Some reports revealed that both Rb1 and Re decreased cardiac contraction in adult rat ventricular myocytes (Scott *et al*. 2001). Some reports says that treatment of both Rg1 and Re could be a novel group of nonpeptide angiogenic agents and may be useful for tissue regeneration (Yu *et al*. 2006). Compared with other gingenosides Re showed anti-diabetic activities (Xie *et al*. 2005) and free radical scavenging properties (Xie *et al*. 2006).

Ginsenoside Rb2: The another form of gingenoside Rb2 is reported to stimulate protein and RNA synthesis and increases RNA polymerase activity (Yokozawa *et al*. 1993a, 1993b,

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Yokozawa *et al*. 1990). Rb2 accelerates would healing effects like cell proliferation, expression of proliferation related factors, and epidermis formation (Choi, 2002). Rb2 can be used as anti-tumor or chemopreventive agents, especially acting on cancer promotional stage (Kang *et al*. 2000).

Ginsenoside Rc: Ginsenoside Rb2 and Rc may have effects that prevent or limit the development of breast cancer, affects the motility of sperm (Chen *et al*. 2001, Lee *et al*. 2003).

Ginsenoside Rd: Ginsenoside Rd inhibiting 26S proteasome activity. Ginsenoside-Rd has been proved to decrease the severity of renal injury induced by cisplatin. Rd is Cytotoxic towards HeLa Cancer Cells and Induces Apoptosis (Chang *et al*. 2008, Yokozawa *et al*. 2000, Yang *et al*. 2006).

Ginsenoside Rh2: Ginsenoside Rh2 has the anti-fatigue, anti-cancer effect, antiallergic and reduces ischemic brain injury in rats (Oh *et al*.1999, Nakata *et al*. 1998, Park *et al*. 2003, Park *et al*. 2004).

Ginsenoside Rh1: Ginsenoside Rh1 and Rh2 inhibit the induction of nitric oxide synthesis in murine peritoneal macrophages. G-Rh1 acts as a functional estrogenic ligand in MCF-7 cells. Ginsenoside Rh1 possesses antiallergic and anti-inflammatory activities (Park *et al*. 1996, Lee *et al*. 2003, Park *et al*. 2004).

Ginsenoside Rg3: Ginsenoside Rg3 significantly inhibited growth and angiogenesis of ovarian cancer and inhibition of *in vitro* Tumor Cell Invasion (Xu *et al*. 2008, Shinkai *et al*. 1996). Ginsenoside Rg3 is a body function balancer, long term taking Rg3 can get anti

senility effects. Because ginsenoside Rg3 can invigorate blood circulation and improve brain and body activity. It has obvious effects for anti wrinkle, would make skin fresh, bright and glossy. Ginsenoside Rg3 has obvious function for body tonic, improve muscle tone and prevent fatigue.

Due to unavoidable limitations in *Panax ginseng* breeding, it is difficult to produce large amounts of roots under field conditions (Han *et al.* 2009). Hence, several scientists followed different biotechnological methods such as root culture, *Agrobacterium*-mediated hairy root production, bioreactor mediated large scale production. Jasmonic acid has been shown to be an effective elicitor for secondary metabolite induction in plant cell cultures (Ketchum *et al.* 1999). Recently, MJ (Methyl jasmonate) mediates the reprogramming of cellular metabolism and cell cycle progression via the regulation of jasmonic acid biosynthesis (Pauwels *et al.* 2008). The positive effect of MJ on ginsenoside production from ginseng cell suspension, hairy root and adventitious root cultures has been previously documented (Lu *et al.* 2001, Palazón *et al.* 2003, Choi *et al.* 2005, Bae *et al.* 2006). Mutation breeding is considered as one of the effective plant breeding methods for improved variety of crop production. Among several methods, γ-irradiation was used in several species for crop improvement program. Recent report showed that mutagenesis by γ-irradiation gained rapid development in enhanced ginsenoside production of *Panax ginseng*. Callus were used for gamma irradiation and the cell lines selected from dosage 30Gy-treatment and ginsenoside content was significantly increased (Kim *et al.* 2009). Suspension culture of *Lithospermum erythrorhizon* cells was irradiated by gamma irradiation and significantly stimulated the shikonin

biosynthesis of the cells and increased the total shikonin yields by 400% at 16Gy (Chung *et al.* 2006). For maize, grain yield for irradiated samples is increased to levels above the unirradiated yield at doses up to about 250Gy with the optimum yield occurring at 150Gy. The corresponding increase for groundnut is observed at doses up to about 930Gy with optimum yield at a dose of 300Gy (Mokobia *et al.* 2006). Grain yield of maximum production in barley was observed at the rate of 100Gy (Subhan *et al.* 2004). γ-irradiation mediated mutagenesis of whole plants as well as roots yielded significant variations among the different mutated species, and 50Gy of γ-rays was determined to be the optimal dose for inducing mutations (Joseph *et al.* 2004).

My present work is to investigate the effects of gamma irradiation on ginsenoside contents of ginseng adventitious root and to provide valuable information on the utilization of beneficial effects of gamma irradiation in root cell culture system of *Panax ginseng.*

MATERIALS AND METHODS

Plant materials

Panax ginseng is Korean wild ginseng. Wild ginseng grows naturally within its natural habitat conditions (Figure 2). Adventitious roots were induced and proliferated from wild *Panax ginseng* root, and cultured in MS medium supplemented with NAA and IAA.
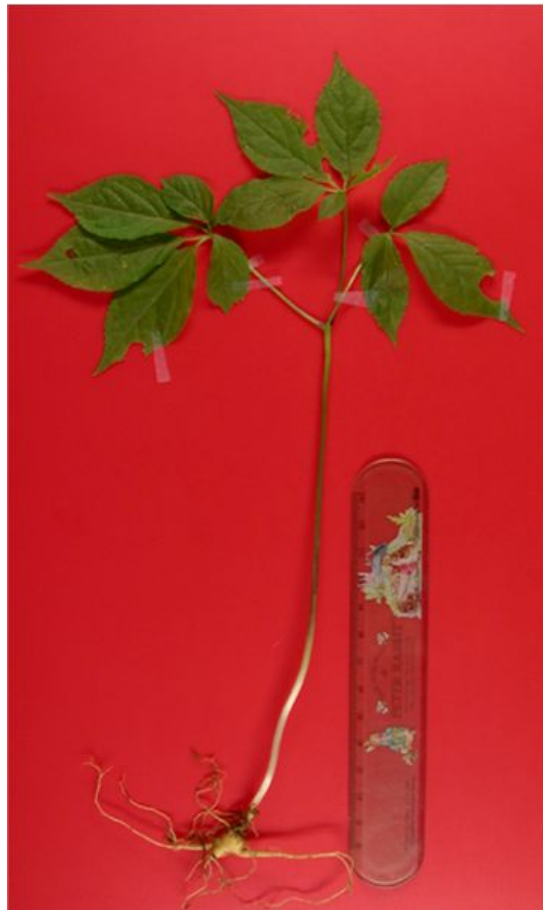
Figure 2. Korean wild ginseng (*Panax ginseng* Meyer).

Establishment of adventitious root cultures

Panax ginseng adventitious roots were proliferated on MS (MS, Murashige and Skoog 1962) medium as follows (mg/L): NH_4NO_3 (687.5), KNO_3 (1900), KH_2PO_4 (170), H_3BO_3 (6.2), $MnSO_4.4H_2O$ (23.3), $ZnSO_4.7H_2O$ (8.6), KI (0.83), $Na_2MoO_4.2H_2O$ (0.25), $CuSO_4.5H_2O$ (0.025), $CoCl_2.6H_2O$ (0.025), $CaCl_2.2H_2O$ (440), $MgSO_4.7H_2O$ (370), Na_2EDTA (37.3), $FeSO_4.7H_2O$ (27.8), Nicotinic acid (5), Pyridoxine Hydrochloride (1), Thiamine Hydrochloride (2.5), Glycine (2). Supplemented with 10.87μmol Naphthalene acetic acid (NAA), 1.43μmol Indole-3-acetic acid (IAA) and 3% sucrose. The pH of the medium was adjusted to 6.0 before autoclaving at 121℃ and 1.2 Kgf/cm^2 pressure for 15 min.

Petridish culture condition

Adventitious roots were cut into 10 pieces, each 1-2 cm and cultured in petridish (10cm in diameter and 1.5 cm in height) with lid containing 50 mL MS solid medium was sealed with a wrap (Advantec, USA) and cultured at 23 ± 2℃ under dark condition. Adventitious roots were sub-cultured every 30 days.

Flask culture condition

Fresh roots (0.8g) were inoculated into a 100 mL erlenmeyer flask containing 50 mL MS liquid medium with 10.87 μmol NAA, 1.43 μmol IAA and 5% sucrose. Cultures were shaken at 110 rpm in the light at 23 ± 2℃ for 30 days.

Bioreactor culture condition

The bioreactors were maintained at 23 ± 2℃ in a light condition until harvest. Adventitious roots were sub-cultured every 30 days in 15L bioreactor (Biopia, Korea)

containing 5L MS medium. Forty-five g fresh weight of adventitious roots was inoculated

into bioreactor for proliferation. After 30 days, the proliferated adventitious roots were used

as explants for further experiments. The culture system showed in figure 3.
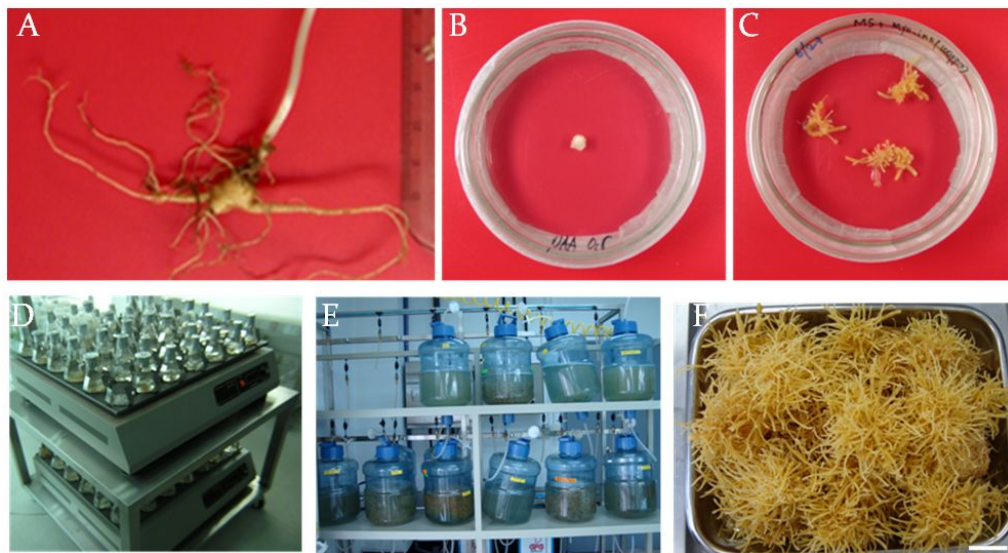
Figure 3. Culture system of *Panax ginseng*. (A) Root of *Panax ginseng*; (B) Explant of

ginseng root; (C) Adventitious root proliferation; (D) Adventitious root culture in flask; (E)

Adventitious root culture in bioreactor; (F) Harvest.

Gamma irradiation

Adventitious roots (1-2cm) were placed in plastic petridishes (10 pieces adventitious roots per pertridish), grown at 23℃ and cultured for 5 days in MS medium with 10.87 µmol NAA, 1.43 µmol IAA and 3% sucrose. They were exposed to gamma radiation from cobalt (^{60}Co) source using a γ-radiation apparatus at the Applied Radiological Science Research Institute, Jeju National University. Irradiation dosages were 0 (non-irradiated), 25, 50, 75, 100 and 125Gy. For each dosage, 3 petridishes of the samples were exposed in triplicate. Effects of gamma irradiation on survival rate of adventitious roots were evaluated by measuring the number of survival adventitious roots after 5 weeks culture.

Selection of cell lines from suspension culture

Secondary roots of survival main roots were transferred into 50 mL liquid MS medium with NAA and IAA in flask. Cell line was selected according to the secondary roots number, length, diameter, growth ratio.

Determination of root weight and growth ratio

Fresh weight (FW) and dry weight (DW) were measured after 30 days growth in flask. FW and DW of roots were determined as follows. Roots were separated from the medium by passing through a 1 mm stainless steel sieve. Root FW was measured after rinsing once with tap water and blotting away surface water and root DW was recorded after roots were dried to a constant weight at 38℃ for several days (Kim *et al.* 2004). Root growth ratio was

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calculated by using the following formula (Yu *et al.* 2002):

$$Growth \ ratio = \frac{Harvested \ DW \ (g)}{Inoculated \ DW \ (g)}$$

Extraction of crude saponin

Extraction and determination of ginsenosides were carried out by modifying the method of Kwon *et al.* (2003). Ultrasound-assisted extraction was performed with an ultrasonic water bath (Branson ultrasonics, USA). The output power is 117 volts and the frequency is 50/60Hz. Sample powder 0.5 g was placed into a 100 mL conical flask, into which 30 mL of 80% (v/v) methanol–water were added. Then the flask was sonicated for 1 h in an ultrasonic water bath. The extract obtained was evaporated using a rotary evaporator under vacuum at 55℃. The evaporated residue (total extract yield) was dissolved in 20 mL of distilled water and washed twice with 20 mL of diethyl ether to remove the fat contents using a separatory funnel. The aqueous layer was extracted four times with 20 mL of water-saturated n-butanol. The butanol solution was washed twice with 30 mL of distilled water to remove the impurities, thereby obtaining crude saponins. The remaining butanolic solution was transferred to the tarred round bottom flask for the evaporation using a rotary evaporator under vacuum at 55℃ (Figure 4).
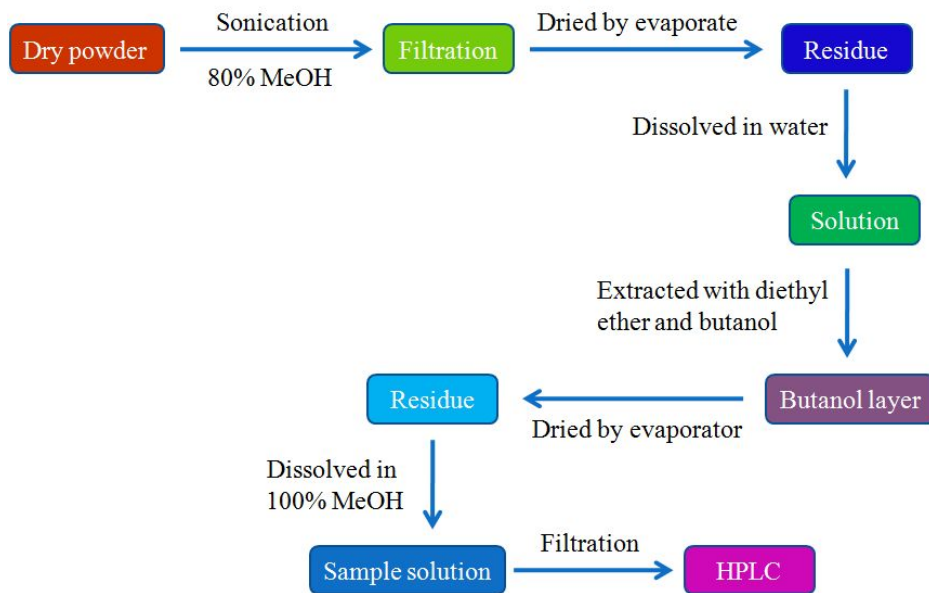
Figure 4. Extraction procedures of crude saponin in *Panax ginseng*.

Determination of ginsenoside content by HPLC

The HPLC conditions for ginsenoside assay was slightly modified the previous report (Park *et al*. 2007). Quantitative determinations were achieved by HPLC using a Capcell-pak C18 MG (4.6 × 250 mm) column (Shiseido, Japan), Waters 2998 Photodiode Array Detector, Waters 2690 Separations Module and Empower Program (Table 1).

Table 1. HPLC conditions for ginsenosides analysis.

Parameter	Condition
Instruments	Waters 2690 Separations Module Empower Program
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	192 - 400 nm
Column temperature	35℃
Sample injection	10 μL
Run time	60 min

The solvents of HPLC grade were used. The water used in this study was treated with a Milli-Q water purification system (Millipore, USA). The mobile phase of HPLC gradient was conducted as follows (Table 2). Ginsenosides Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd standards were purchased from BTGin Co., Ltd (Daejeon, Korea). Digoxin was used as internal standard.

Table 2. Mobile phase of HPLC gradient condition for ginsenosides analysis.

Retention time (min)	Solvent (A)	Solvent (B)
0	82	18
22	70	30
32	55	45
50	50	50
55	82	18
60	82	18

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

Stock solutions for the 4 ginsenosides were prepared separately in 100% MeOH. Digoxin stock solution was prepared in 70% MeOH. Working solutions were prepared in methanol by mixing known amount of all the ginsenosides together. Five concentrations were made for standard curves, each concentration was 60, 120, 240, 320, 480 ppm. Ginsenosides were detected at a wavelength of 203 nm with the peak areas corresponding to ginsenosides from the samples matching retention times as authentic ginsenoside standards.

Analysis of ginsenosides 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)

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

Method validation and statistical analysis

Stock solutions for the 8 ginsenosides were prepared separately in 100% methanol.

Digoxin stock solution was prepared in 80% methanol. Working solutions were prepared in

100% methanol by mixing known amounts of all the compounds. The linear range, limit of

detection (LOD) and limit of quantification (LOQ) were studied for the developed method.

The linearity of calibration curve was tested by standard analysis. The calibration curves of

individual ginsenosides were constructed using a range of five concentrations of the standard,

and LOD and LOQ for each analyte were evaluated at signal-to-noise ratios (S/N) of 3:1 and

10:1, respectively.

Statistical analysis was performed according to the SPSS system. Mean and standard errors

were used throughout and statistical significance between the mean values was assessed by

applying a Duncan's multiple range tests. A probability of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Effects of gamma irradiation on survival rate of adventitious roots

In this experiment, I first determined the survival rate of adventitious roots at different dosage of γ-irradiation. The survival rates of adventitious roots declined with increasing dosage of irradiation (Figure 5). No survived adventitious roots were observed at 125Gy. The growth of adventitious roots is inhibited over 50Gy treatment and the LD50 was established by 30Gy irradiation of adventitious root based on the survival rates of main root.

Panax ginseng was irradiated by ^{60}CO gamma ray and the growth of hairy roots was inhibited over 30Gy (Choi *et al*. 2002). LD50 was established by irradiation of shoot tip explants at 30Gy based on the survival of explants and shoot proliferation (Ali, 2006).

Effects of gamma irradiation on growth rate of cell lines

Five cell lines were selected at last according to the phenotype (Figure 6). After 30 days culture in flask, dry weight of control was 0.31g and the selected cell lines CL1, 3, 9, 13 and 14 were 0.46g, 0.45g, 0.29g, 0.34g and 0.48g respectively. The value of growth ratio of control was 4.42 and the selected cell lines CL1, 3, 9, 13, 14 were 6.57, 6.48, 4.1, 4.9 and 6.9, respectively (Figure 7). The values of growth ratio in cell lines CL1, CL3 and CL14 were increased 49%, 47% and 57%, respectively, compared to the control.

Panax ginseng was irradiated by gamma ray and cell lines were selected according to the higher growth ratio at 30Gy (Kim *et al*. 2009). *Lithospermum erythrorhizon* S. was irradiated

by gamma ray and the cell lines selected in terms of growth rate (similar with control) and appearance of shikonin (Chung *et al.* 2006). In this experiment, cell line CL1, 3 and 14 showed higher growth ratio.

Effects of gamma irradiation on ginsenoside production

The spectrum of Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd were 203nm. The calibration curves and the LOD for the ginsenosides are shown in Table 3 and Figure 8. The correlation coefficients are all better than 0.99, which show good linearity.

The chromatograms for 8 major ginsenosides among each cell line and the internal standard shown in Figure 9, 10, 11, 12 and 13. The contents of the eight ginsenosides were significantly enriched, especially in the cell line CL1 (Figure 14). The total contents of ginsenosides (Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd) in cell line CL1 were 1.6-fold higher than that of control. In the cell line CL1, the level of ginsenoside Rg1, Re, Rh1and Rb2 contents were increased by 2-3 times than that of the control. In the cell line CL3, level of ginsenoside Re, Rh1, Rb2, Rc and Rd contents were increased by 1.6-2.3 times than that of the control. In the cell line CL9, level of ginsenoside Rg1, Re and Rh1 contents were increased by 1.7-2 times than that of the control. In the cell line CL14, level of ginsenoside contents were similar with that of the control. In case of the cell line CL13, the ginsenoside contents were affected negatively in ginseng adventitious root cultures. Overall, the highest ginsenoside contents were obtained in cell line CL1.

CONCLUSIONS

In the present study, I investigated the influence of gamma irradiation on the content of ginsenosides of Korean wild ginseng in order to search for better application of ginseng as a health food. As it is known, in root cultures, the ginsenoside yield depends not only on the accumulation of the commercially useful compounds but also on their biomass production capability. In this study, I selected cell line CL1 as the best cell line. Because of the ginsenoside content and the growth ratio of the CL1 were highest compared to control and other cell lines. So, the ginsenoside productivity of CL1 is better than other cell lines. In the next study, I will use these mutant cell lines to establish the best condition for regeneration system of *Panax ginseng* and this system also use for genetic transformation.
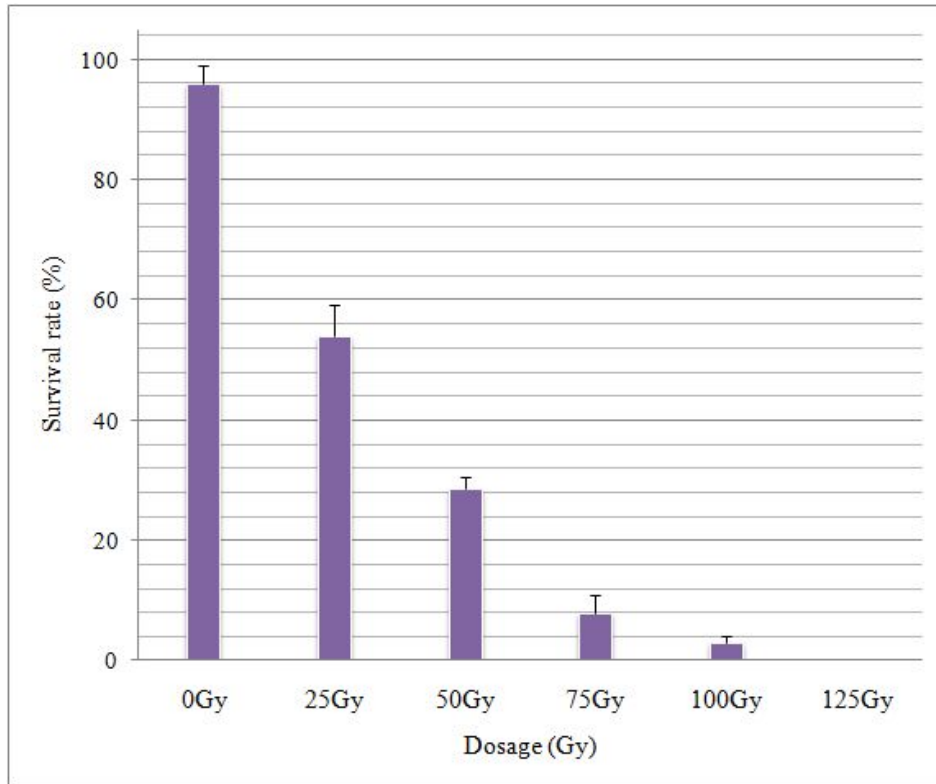
Figure 5. Survival rate among gamma-ray irradiated adventitious roots of *Panax ginseng*.

After irradiation, the adventitious roots were cultured in the petridish for 5 weeks on MS

medium with NAA, IAA and 3% sucrose at 23℃ in light condition. Bar shown are means ±

standard deviations of three replicates.
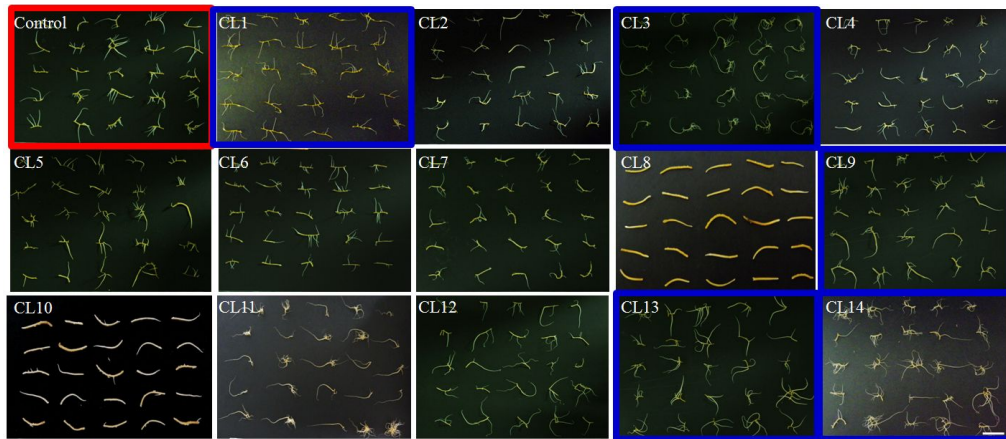
Figure 6. Phenotypic characteristics of secondary roots in the gamma-irradiated cell lines of *Panax ginseng* (scale bar = 1 cm). Control: non-irradiated adventitious roots, CL1-14: gamma-irradiated mutant cell lines.
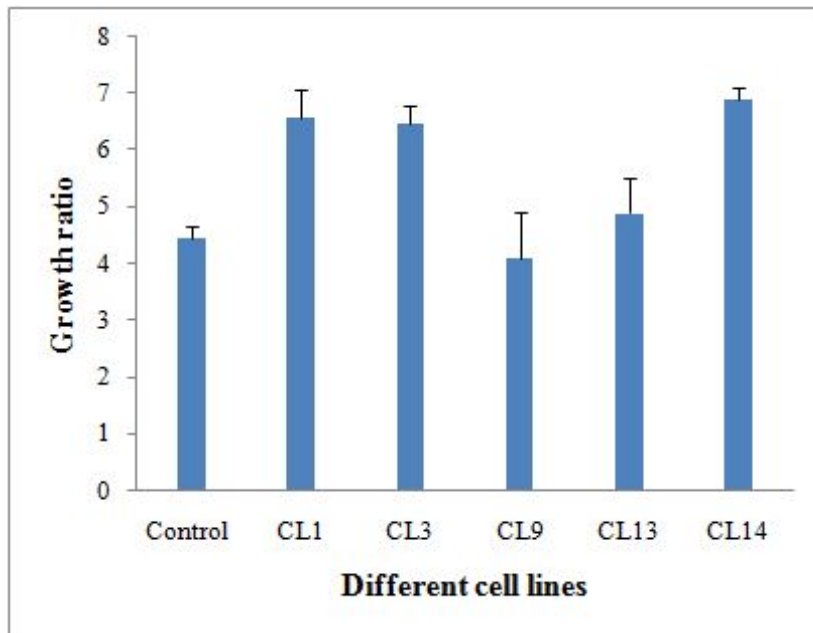
Figure 7. Growth ratio of *Panax ginseng* adventitious root in the control and gamma-irradiated cell lines. The adventitious roots were cultured in the flask for 30 days with 50 mL MS medium. Bar shown are means ± standard errors of three replicates. Control: non-irradiated adventitious roots, CL1-14: selected mutant cell lines.

Table 3. Calibration curve, limit of detection (LOD), and limit of quantification (LOQ) for

eight ginsenosides.

Compound	Calibration curve[a]	R^2	Linear range (mg/L)	LOD (mg/L)	LOQ (mg/L)
Rg1	Y = 3762.2X-719.38	0.9908	25-150	2.32	7.71
Re	Y = 2452.5X-22171	0.9979	30-180	1.0	3.06
Rf	Y = 9588.1X-10035	0.9995	10-50	0.45	1.51
Rh1	Y = 14271X-32882	0.9987	10-50	0.32	0.98
Rb1	Y = 2343.7X-1448.4	0.9967	30-150	0.84	2.81
Rc	Y = 6048.9X-12290	0.9984	10-50	0.5	1.67
Rb2	Y = 1978.3X-27044	0.9933	50-200	1.30	4.32
Rd	Y = 6263X-1238.8	1	10-50	0.08	0.27

[a]y is the peak area and x is the concentration of analyte.
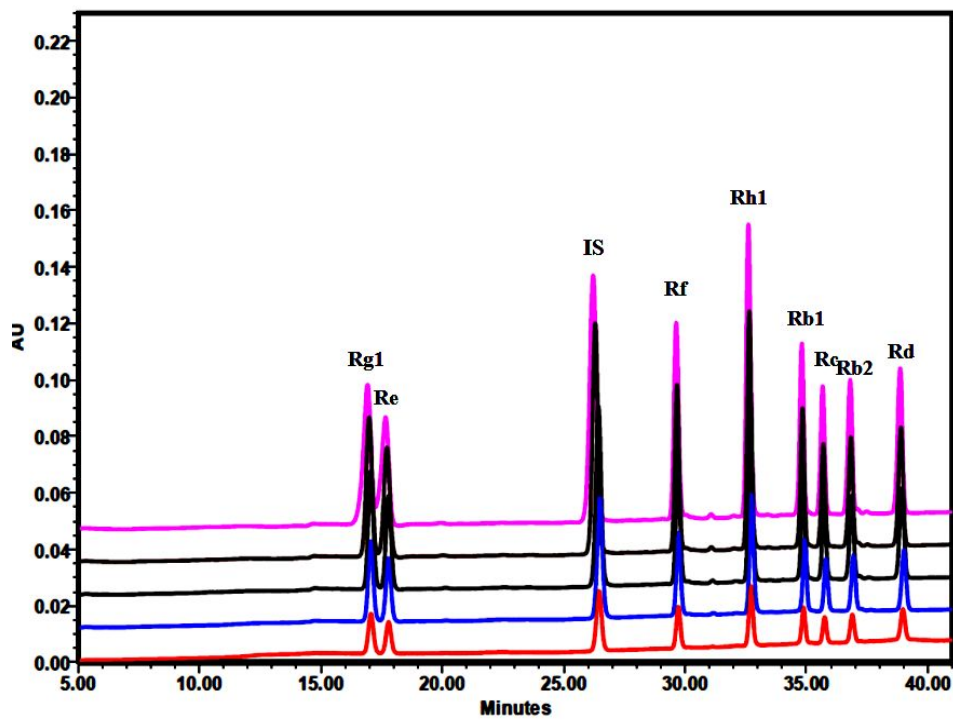
Figure 8. Typical chromatograms obtained from the standard solution by UV detection at 203 nm. IS: internal standard (digoxin).
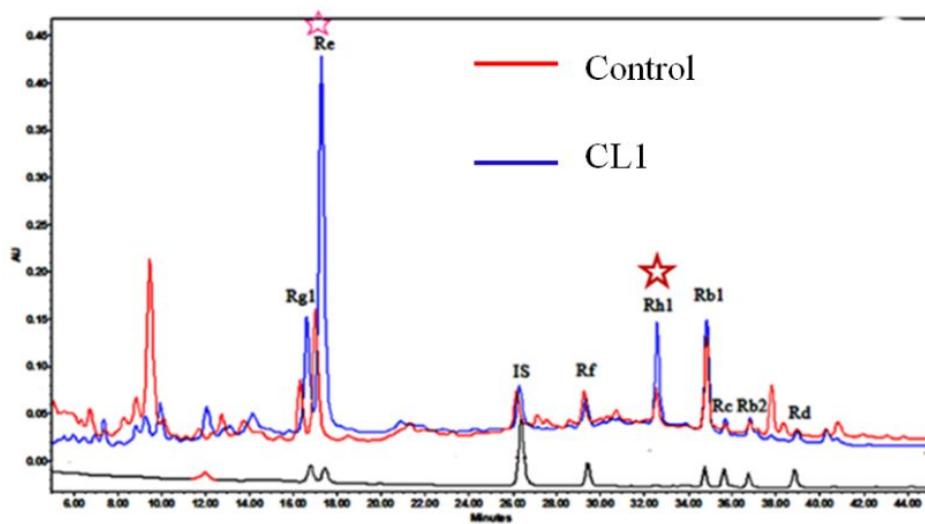
Figure 9. Typical chromatograms and ginsenoside content obtained from the standard, control and cell line CL1. The ginsenosides were extracted from adventitious roots cultured in the flask. Control: non-irradiated adventitious root, CL1: selected cell line 1 from gamma-irradiated adventitious roots, IS: internal standard (digoxin).
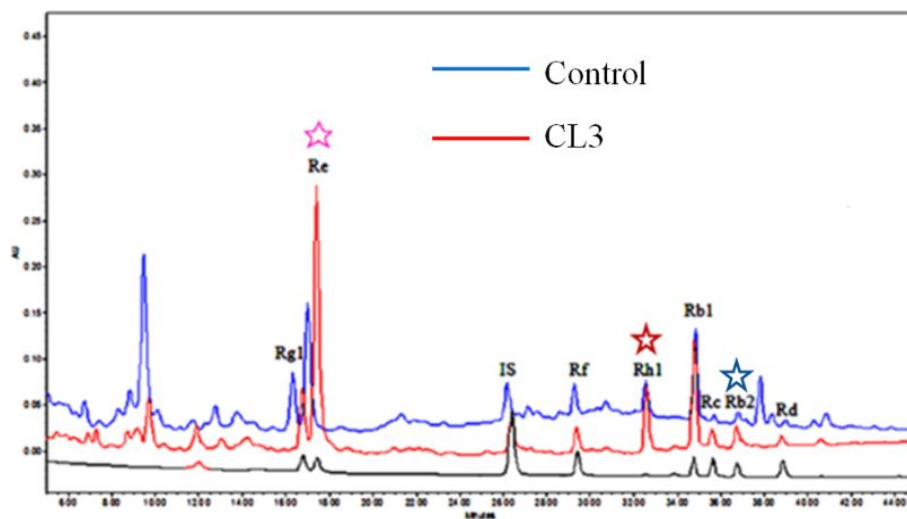
Figure 10. Typical chromatograms and ginsenoside content obtained from the standard, control and cell line CL3. The ginsenosides were extracted from adventitious roots cultured in the flask. Control: non-irradiated adventitious root, CL3: selected cell line 3 from gamma-irradiated adventitious roots, IS: internal standard (digoxin).
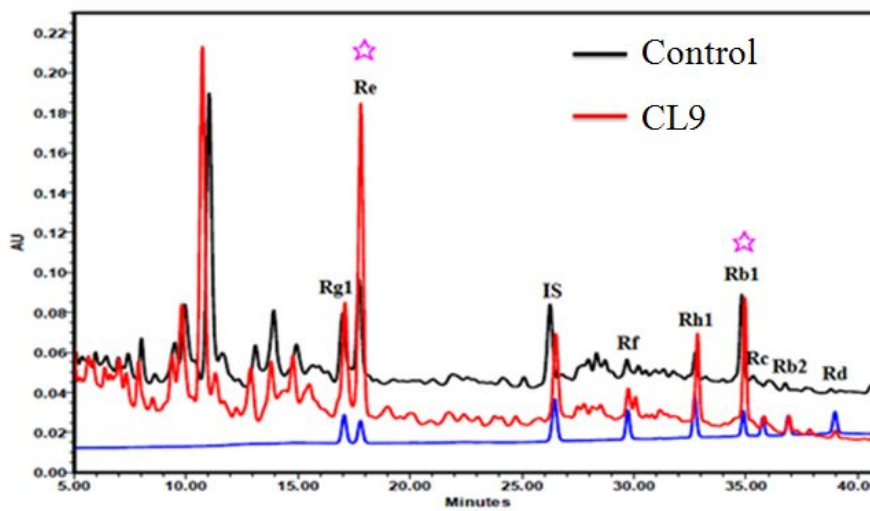
Figure 11. Typical chromatograms and ginsenoside content obtained from the standard, control and cell line CL9. The ginsenosides were extracted from adventitious roots cultured in the flask. Control: non-irradiated adventitious root, CL9: selected cell line 9 from gamma-irradiated adventitious roots, IS: internal standard (digoxin).
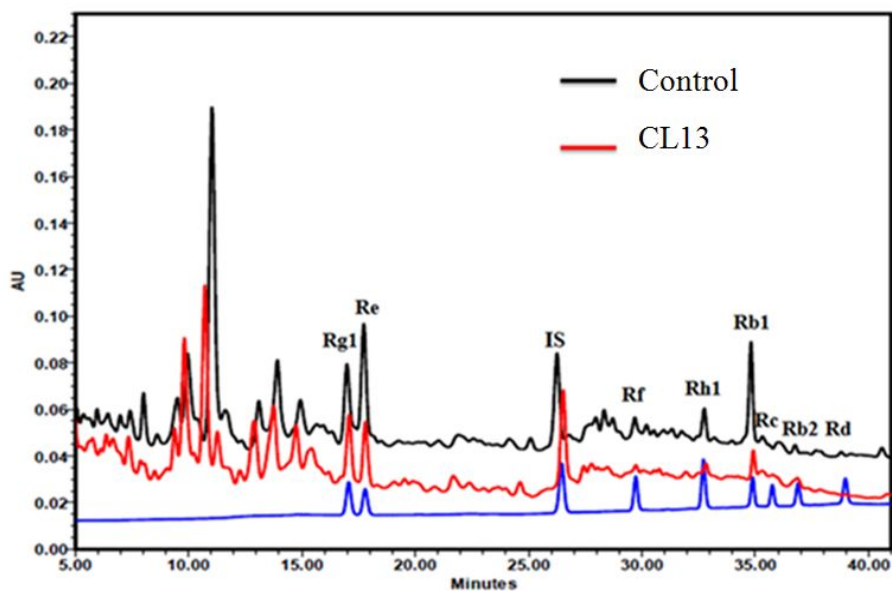
Figure 12. Typical chromatograms and ginsenoside content obtained from the standard, control and cell line CL13. The ginsenosides were extracted from adventitious roots cultured in the flask. Control: non-irradiated adventitious root, CL13: selected cell line 13 from gamma-irradiated adventitious roots, IS: internal standard (digoxin).
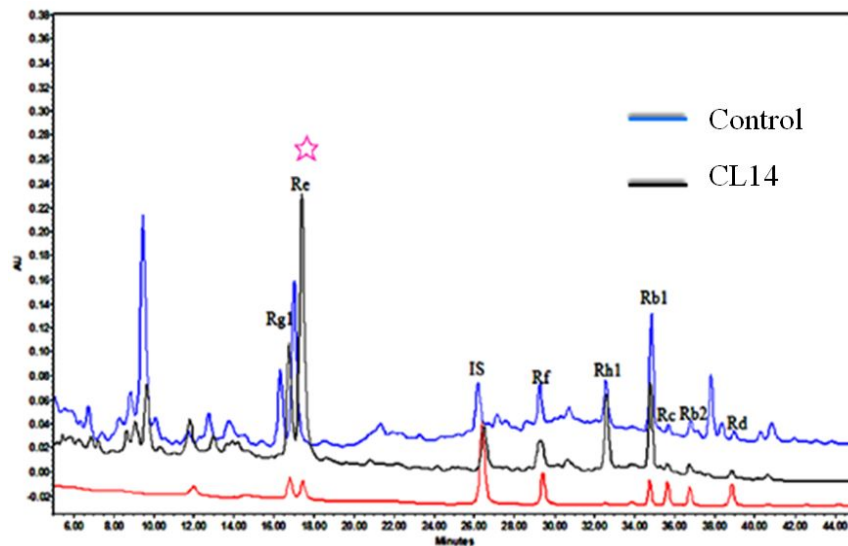
Figure 13. Typical chromatograms and ginsenoside content obtained from the standard, control and cell line CL14. The ginsenosides were extracted from adventitious roots cultured in the flask. Control: non-irradiated adventitious root, CL14: selected cell line 14 from gamma-irradiated adventitious roots, IS: internal standard (digoxin).
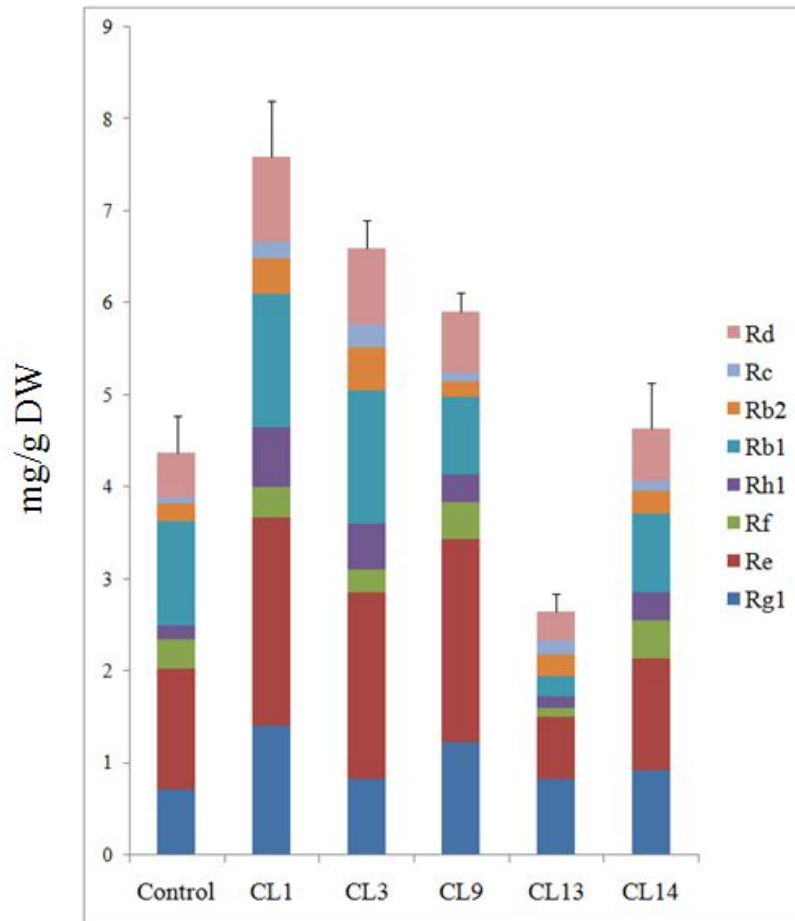
Figure 14. Total ginsenoside content of the control and mutant cell lines of *Panax ginseng*

adventitious root. Control: non-irradiated adventitious root, CL1-14: selected cell lines from

gamma-irradiated adventitious roots.

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Chapter II

Plant regeneration of Korean wild ginseng (*Panax ginseng* Meyer) mutant lines induced by γ-irradiation (^{60}Co) of adventitious roots

INTRODUCTION

Panax ginseng Meyer is an important medicinal herb and is widely cultivated in Korea, China and Japan. The root has been used as a drug for over 2000 years in oriental countries. Its use is rapidly expanding in the Western countries as complementary and alternative medicine (Shim *et al*. 2007). Ginsenosides are the major pharmacologically active components in *P. ginseng*. More than 30 types of ginsenosides have been identified from the genus (Harrison *et al*. 1990, Nah *et al*. 1997).

Ginseng is a perennial plant which grows slowly and has a long production cycle (4-6 years). And more than 3 years of juvenile period are required for producing seeds (Ahn *et al*. 1996, Choi *et al*.1998). This has made the generation of superior genotypes by conventional breeding very difficult. Because of these reasons, attempts have been made to achieve a more rapid and increased production of the ginsenosides using other methods such as classical tissue culture system (Wu *et al*. 2005), bioreactor culture system (Sivakumar *et al*. 2005), *Agrobacterium*-mediated hairy root production (Yoshikawa *et al*. 1987, Mallol *et al*. 2001), using elicitors in cell cultures (Lu *et al*. 2001, Palazon *et al*. 2003, Bae *et al*. 2006), and mutation breeding by γ-irradiation (Kim *et al*. 2009, Kim *et al*. 2013). The last method has been used in many other plant species and has provided a large number of variants useful for plant breeding (Subhan *et al*. 2004, Mokobia *et al*. 2006, Chung *et al*. 2006). Mutagenesis by γ-irradiation enhanced ginsenoside production in *P. ginseng* (Kim *et al*. 2009, Kim *et al*. 2013). Recently, I have also generated mutant cell lines by applying

γ-irradiation on *P. ginseng* adventitious roots which were derived from Korean wild ginseng root (Zhang *et al*. 2011). Among the selected mutant cell lines, line 1 has showed the highest total ginsenoside content of 7 major ginsenosides (Rg1, Re, Rb1, Rb2, Rc, Rf, and Rd). The total ginsenoside content of the mutant line was 2.3 times higher than in the wild-type line (Zhang *et al*. 2011). Using γ-irradiation, I have created a useful mutant line for breeding of the ginseng plant. However, there are no reports on in vitro plant regeneration with mutant lines of ginseng adventitious root.

Plant tissue culture system is considered a valuable tool in the plant improvement program. Somatic embryogenesis has been used as a preferred method for rapid in vitro propagation of many plant species (Guerra *et al*. 1988, Bhansali *et al*. 1990, Jimenez *et al*. 2005). *P. ginseng* is a difficult species to manipulate in vitro; however, its regeneration has generally been accomplished using somatic embryogenesis in callus derived from mature root tissues (Chang *et al*. 1980, Cellarova *et al*. 1992, Lim *et al*. 1997), callus derived from zygotic embryo (Lee *et al*. 1990, Arya *et al*. 1993), protoplast derived from callus (Arya *et al*. 1991), and cotyledons (Ahn *et al*. 1996, Choi *et al*. 1998, Choi *et al*. 1999, Kim *et al*. 2012). The development of efficient *in vitro* culture methods has facilitated the use of mutation technique for improvement of vegetative propagation of ginseng adventitious roots (Kim *et al*. 2009, Kim *et al*. 2013, Zhang *et a*l. 2011). At present no information is available on the regeneration of mutant adventitious root line that has been selected from γ-irradiated *P. ginseng* adventitious roots.

In this chapter, I report on an efficient procedure for the regeneration of wild-type and

mutant cell lines of *P. ginseng* adventitious roots through somatic embryogenesis.

MATERIALS AND METHODS

Callus induction and proliferation

Adventitious roots derived from Korean wild ginseng were provided by Sunchon National University. The adventitious roots were generated as described previously (Sivakumar *et al*. 2005, Yu *et al*. 2002, Kim *et al*. 2003) and have been maintained in my laboratory for over 10 years. A mutant adventitious root line has been generated from the wild type adventitious roots by γ-irradiation (Zhang *et al*. 2011). For embryogenic callus induction, wild-type and mutant adventitious roots were sectioned into 10 mm in length and were placed on MS solid medium supplemented with 2, 4-D, kinetin and 3% sucrose. The media were solidified with 0.3% Gelite. Callus induction frequency was tested on MS solid medium supplemented with various concentrations of 2, 4-D (0.5, 1, 1.5, 2 mg/L) and kinetin (0, 0.3, 0.5 mg/L). All media were adjusted to pH 5.8 before autoclaving. Thirty pieces of adventitious roots were placed on each petridish. Three replicates were prepared for each treatment. All cultures were incubated at 25℃ in the dark. Callus formation was observed after 4 weeks of culture. After 6 weeks of culture, the frequency of callus induction was estimated. The induced callus was subcultured at 3 week intervals on the same medium for induction of embryogenic callus and maintenance.

Induction of somatic embryos

Embryogenic callus induced from the segments of adventitious roots was used for

induction of somatic embryos. Ten g of embryogenic callus was incubated in a 15 L airlift

bioreactor containing 5 L MS liquid medium with 0.5 mg/L 2, 4-D and 3% sucrose for

proliferation. After 3 weeks, the proliferated embryogenic callus was used as explants for

induction of somatic embryogenesis.

To examine the effect of 2, 4-D on somatic embryo induction, proliferated callus was

placed on a solid MS medium supplemented with different concentrations of 2,4-D (0, 0.5, 1

mg/L). Ten clumps of embryogenic callus (about 5mm in diameter) were cultured on

petridishes containing 40 mL of medium and the experiment repeated three times. All

cultures were incubated at 25℃ in the dark. The frequency of somatic embryo production

was examined after 6 weeks of culture by counting cultured embryogenic callus that formed

somatic embryos.

When the callus produced globular stage embryos on MS solid medium with 2,4-D and

3% sucrose, the globular embryos were removed and transferred to 500 mL-Erlenmeyer

flasks containing 200 mL of liquid MS medium supplemented with 2,4-D and 3% sucrose

for further growth. The liquid cultures were agitated at 100 rpm on a gyratory shaker in the

dark. After 1 month of culture, the proliferated globular embryos in flasks were transferred

to each petridish containing solid MS medium with GA_3 and 3% sucrose for maturation and

germination of embryos.

Maturation and germination of somatic embryos

The proliferated globular embryos in flasks were transferred to 40 mL MS solid medium

supplemented with GA_3 and 3% sucrose in 100×20 mm plastic petridishes for maturation and germination. To investigate the effect of GA_3 concentration on maturation and germination of somatic embryos, 150 globular embryos were transferred to germination medium containing 0, 1, 3, 5, 7, or 10 mg/L of GA_3. Cultures were maintained at $23 \pm 2\,℃$ under dim light illumination (12 μmol m^{-2} s^{-1}) with a 16/8 h (light/dark) photoperiod. After 6 weeks of culture, maturation and germination of embryos were examined. The experiment was repeated three times.

Development of plantlets and acclimatization

When shoots reached 0.5-1.0 cm in height, the plantlets were transferred from germination medium to elongation medium, 50 mL MS solid medium supplemented with 5 mg/L GA_3 in 100×40 mm plastic petridishes, for shoot elongation. When shoots grew 3.0-4.0 cm in height, they were transferred to rooting medium, half or one-third strength MS, or SH basal medium supplemented with 0.25 mg/L NAA or with 0.5% activated charcoal, in 75×130 mm glass bottles, one shoot per bottle. Cultures were conducted in a culture room and maintained in a 16/8 h (light/dark) photoperiod with white fluorescent light (30 μmol m^{-2} s^{-1}) at $23 \pm 2\,℃$.After 4 weeks, the results of rooting were examined.

Plantlets with both shoots and roots were transferred to plastic pots (10×18cm) containing an artificial soil mixture of peatmoss, vermiculite and perlite (2:3:1 v/v) and covered with a transparent polyvinyl film. The potted plants were cultivated in a growth room (40 μmol m^{-2}s^{-1}, 16 h photoperiod, $25 \pm 1\,℃$). After 3 weeks, the plants were hardened

by removing the polyvinyl film gradually on a daily basis for one week, and then the film was removed. After 3 months of culture, the survived plants without wilting were counted. The acclimated plants were transplanted to glasshouse conditions or kept in the growth room for another 4-6 months.

Data analysis

Each of the treatments was performed three times. Statistical analyses were performed according to the one-way analysis of variance (ANOVA) using SPSS software (version 17.0) to assess significant differences in the mean values of different treatments. Comparisons between the mean values were assessed using Duncan's multiple-range test ($P < 0.05$).

RESULTS AND DISCUSSION

Induction of callus from adventitious roots

Initiation of callus from adventitious root explants generally occurred after 3 weeks on media supplemented with different combinations of growth regulators. The highest frequency of callus induction was observed on the medium containing 0.5 mg/L 2,4-D and 0.3 mg/L kinetin. The frequency of callus induction reduced dramatically as the concentration of 2, 4-D increased. Callus was not induced in the presence of 2 mg/L 2,4-D (Table 4; Figure 15). Similar results were reported with the cultures of hairy roots of *P. ginseng* that 2,4-D at more than 3 mg/L strongly suppressed callus induction (Kwon *et al*. 2003). When the segments of adventitious roots (Figure 16A) of *P. ginseng* were incubated in MS solid medium with 0.5 mg/L 2,4-D and 0.3 mg/L kinetin, callus was induced from the cut sides of the adventitious roots after 6 weeks of culture (Figure 16B). The callus was subcultured on the same medium at 3-week subculture intervals. After three months, embryogenic callus was induced (Figure 16C) and the embryogenic callus showed high regenerative capacity and differentiated into somatic embryos and plantlets. Callus induction and growth from adventitious root explants was dependent upon 2,4-D as previously reported (Chang *et al*. 1980, Lim *et al*. 1997, Arya *et al*. 1991). When embryogenic callus was transferred to MS medium lacking kinetin, a small number of globular embryos formed after 3 weeks of culture (Figure 16D, E). Thus, it is essential to induce and maintain the embryogenic callus in the medium supplemented with 2,4-D in combination with kinetin.

Embryogenic callus has been maintained in the dark for more than 2 years through 3-week

subculture intervals on MS solid medium with 0.5 mg/L 2,4-D and 0.3 mg/L kinetin.

Induction of somatic embryos

The embryogenic callus grows better in a liquid medium than a solid medium (data not

shown). Therefore, we propagated the embryogenic callus in bioreactor to assess somatic

embryo development and plantlet conversion. When embryogenic callus was inoculated into

a 15 L airlift bioreactor containing 5 L MS liquid medium with 0.5 mg/L 2, 4-D, the

embryogenic callus was propagated and a small number of globular shaped embryos were

also formed after 3 weeks of culture (Figure 16D, E). The growth rate (final explant fresh

weight/initial explant fresh weight) was about 1.8. Embryogenic cell clumps proliferated in

bioreactor were transferred onto MS solid medium with different concentrations of 2,4-D (0,

0.5 or 1.0 mg/L) for embryogenesis. The frequency of somatic embryo formation was

significantly depended on the concentrations of 2, 4-D (Table 5; Figure 17). The highest

induction frequency of somatic embryos was observed on the medium supplemented with

0.5 mg/L 2, 4-D. The frequency of somatic embryo formation in wild-type and mutant cell

line was15.3% and 14.7%, respectively. The number of somatic embryos per callus was 25.6

and 23.7 in wild-type and mutant cell line, respectively. There was no significant difference

in somatic embryo formation frequency between wild-type and mutant cell line (Table 5;

Figure 17). Globular shaped somatic embryos formed on the surfaces of embryogenic callus

(Figure 16F, G).

Maturation and germination of somatic embryos

These somatic embryos were transferred into 500 mL-Erlenmeyer flasks containing 200 mL of liquid MS medium supplemented with 0.5 mg/L 2,4-D and 3% sucrose(Figure 16H) for proliferation. The growth rate (final explant fresh weight/initial explant fresh weight) was about 1.5. After 4 weeks of culture, the proliferated globular embryos were transferred to petridishes containing solid MS medium with various concentrations of GA_3 and 3% sucrose. At 5 mg/L GA_3, most of the globular embryos turned green and increased in size and developed into torpedo and cotyledonary stage embryos within one month. When the mature somatic embryos were transferred to a fresh medium with the same composition, most of the embryos germinated within 2 weeks of culture (Figure 16I). Adventitious shoots were induced from the mature somatic embryos. The optimal concentration of GA_3 in germination medium was 5 mg/L, yielding the highest germination frequency of 85%. Without GA_3 treatment, the germination frequency was lowest at 36%. Maturation and germination of embryos were strongly influenced by the GA_3 concentration (Table 6; Figure 18). This result suggests that GA_3 is required for maturation and germination of somatic embryos. Similar results were observed in *Eleutherococcus senticosus*, that GA_3 treatment was necessary to induce germination from somatic embryos (Choi *et al*. 1999). GA_3 treatment is also commonly used for maturation and germination of somatic embryos from *P. ginseng* (Chang *et al*. 1980, Arya *et al*. 1993, Choi *et al*. 1998, Choi *et al*. 1999), from *P. quinquefolius* (Zhou *et al*. 2006) and from *P. japonicus* (You *et al*. 2007).

Development of plantlets and transplantation

When shoots reached 0.5-1.0 cm in height on germination medium, the shoots were transferred to elongation medium, 50 mL MS solid medium supplemented with 5 mg/L GA_3 in 100×40 mm plastic petridishes, for further growth of shoots. After about one month of culture, the shoots developed to 3.0-4.0 cm in height, but most of the shoots had no visible roots. The shoots without roots were excised and transferred to different rooting media, half or one-third strength MS, or SH basal medium supplemented with 0.25 mg/L NAA or with 0.5% activated charcoal, in 75×130 mm glass bottles, one shoot per bottle. Adventitious roots formed from the excised regions of the shoots. After 1 month, the rate of root formation from the shoots was examined (Table 7). As far as root quality is concerned, 1/3 SH medium with 0.25 mg/L NAA and 1% sucrose showed the best result among the tested rooting media; the roots grew fast and thickened on the medium(Figure 16J; Figure 19A, B; Table 7). Although 1/3 SH medium with 2% sucrose and 0.5% activated charcoal was most effective in inducing roots, the roots grew well but weak (Table 7). The optimal medium for rooting is therefore 1/3 SH medium supplemented with 0.25 mg/L NAA and 1% sucrose among the tested rooting media in this study. In our comparative studies, SH medium was more effective than MS medium in root induction and proliferation. A very similar result was reported in American (Zhou *et al*. 2006) and Korean ginsengs (Kim *et al*. 2012). It was reported that the high level of ammonium nitrate in MS medium highly suppressed root development in carrot (Halperin *et al*. 1966). Choi et al reported that when the ammonium nitrate was omitted in MS medium, root growth of regenerated ginseng plants was enhanced.

The concentration of ammonium nitrate in SH medium was about 8 times lower than in MS medium. It seems that the different concentrations of ammonium nitrate in SH and MS medium may result in the different root induction efficiency between the two basal medium. From these observations, I suggest that SH medium, especially 1/3 strength SH medium is suitable for root induction and growth of regenerated ginseng plants.

Well-developed plantlets with both shoots and roots derived from adventitious roots were transferred to plastic pots (10×18cm) containing an artificial soil mixture of peatmoss, vermiculite and perlite (2:3:1 v/v) in a growth room (Figure 20C). The survival rate of the plantlets was about 30% after 3 months of culture and new leaf began growing (Figure 19D). The plants regenerated from both wild-type and mutant cell line acclimatized in the growth room (Figure 20).

CONCLUSIONS

I developed an efficient *in vitro* regeneration protocol for an important medicinal plant of *P. ginseng*. The protocol described here will allow a relatively rapid mass production of Korean wild ginseng plants. It takes 6-8 months from the callus induction of adventitious roots to the plantation of plants. In this study, I also produced the regenerated plants from the mutant adventitious roots which were obtained by γ-irradiation. The combination of mutation technique by γ-irradiation and plant regeneration by tissue cultures may be an effective way to ginseng improvement. The protocol established in this study was used for the genetic transformation of this species.
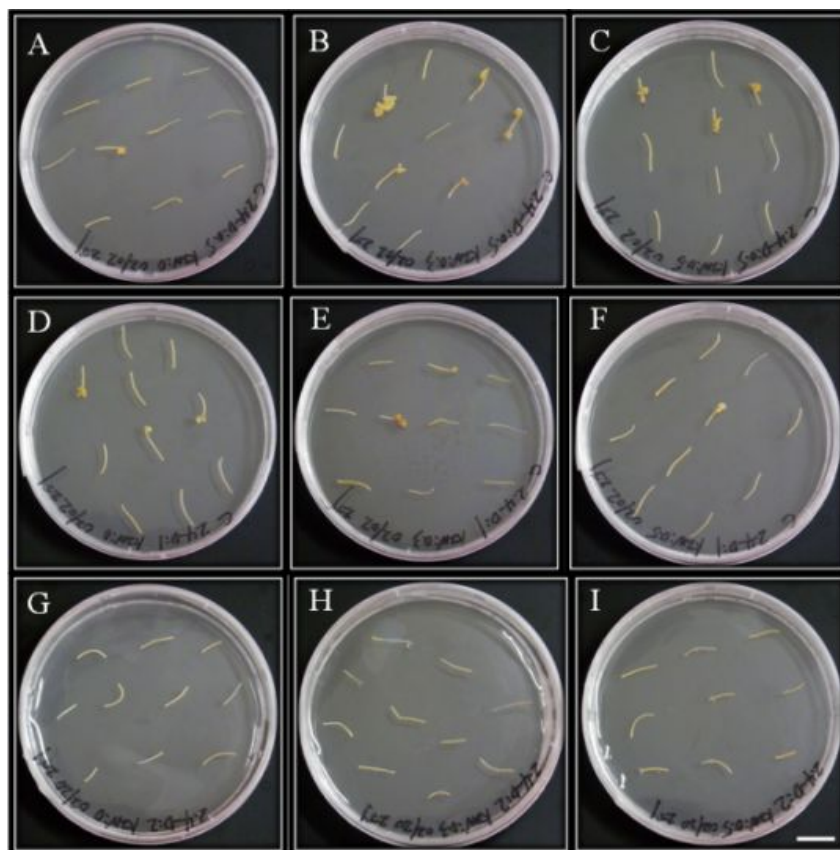
Figure 15. Effects of different concentration of 2,4-D and Kinetin on the frequency of callus formation from ginseng adventitious roots after 6 weeks of a culture (scale bar = 1 cm). A: 2,4-D: 0.5, Kinetin: 0. B: 2,4-D: 0.5, Kinetin: 0.3. C: 2,4-D: 0.5, Kinetin: 0.5. D: 2,4-D: 1, Kinetin: 0. E: 2,4-D: 1, Kinetin: 0.3. F: 2,4-D: 1, Kinetin: 0.5. G: 2,4-D: 2, Kinetin: 0. H: 2,4-D: 2, Kinetin: 0.3. I: 2,4-D: 2, Kinetin: 0.5.

Table 4. Effects of 2,4-D and kinetin on the frequency of callus formation from ginseng

adventitious roots.

2, 4-D (mg/L)	Kinetin (mg/L)	Number of root explants	Number of root explants formed callus
0.5	0	30	4.3 ±1.0de
	0.3	30	16.2 ± 1.8a
	0.5	30	9.1± 1.7de
1	0	30	7.2 ± 1.2bc
	0.3	30	5.4 ± 1.1cd
	0.5	30	2.3 ± 0.8ef
2	0	30	0.0 ± 0f
	0.3	30	0.0 ± 0f
	0.5	30	0.0 ± 0f

The data were collected after 6 weeks of culture.

The results represent the means ± SEM of values obtained from three experiments.

Different corresponding letters within a column are significant different at $P<0.05$ by

Duncan's multiple range test.
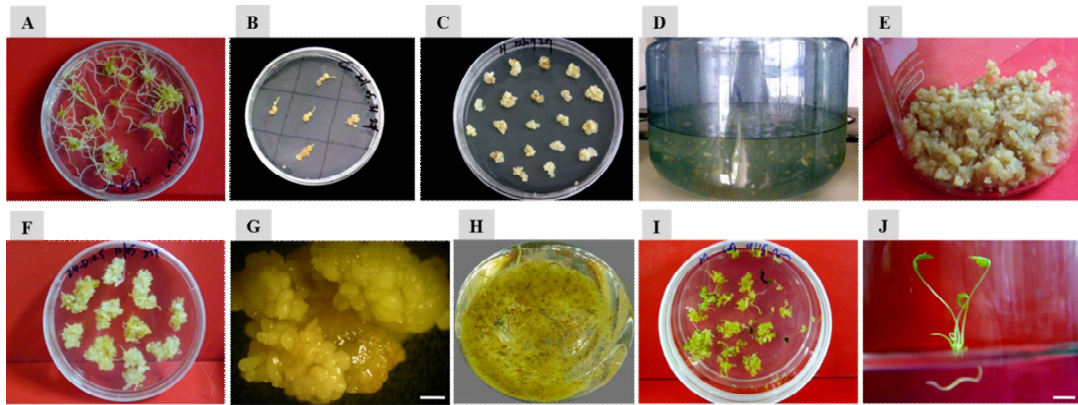
Figure 16. Somatic embryogenesis and regeneration of plantlet from adventitious roots of *Panax ginseng*. (A) Adventitious roots derived from Korean wild ginseng root. (B) Callus induction from adventitious root explants. (C) Embryogenic callus derived from adventitious roots. (D) Proliferation of embryogenic callus in an airlift bioreactor. (E) Proliferated embryogenic cell clumpsfrom bioreactor culture. (F)Somatic embryos formed on embryogenic callus. (G) Magnified image from (F) (scale bar = 2 mm). (H) Proliferation of somatic embryos in conical flasks. (I) Maturation and germination of somatic embryos on MS medium supplemented with 5 mg/L GA_3. (J) Well-developed plantlet derived from somatic embryo (scale bar = 0.8 cm).
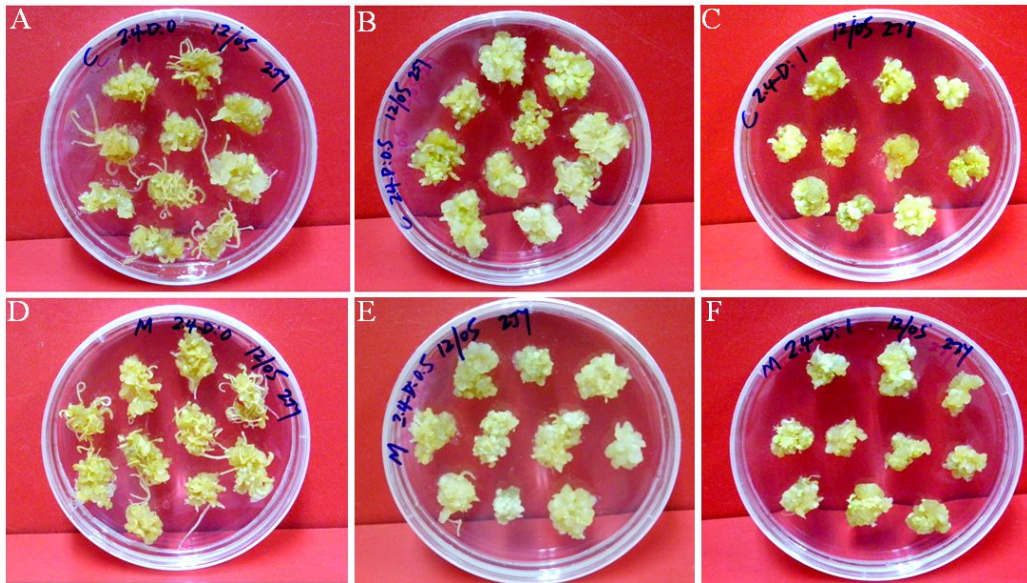
Figure 17. Effect of 2,4-D on somatic embryogenesis from embryogenic callus of control and mutant cell line after 6 weeks of culture. A (control): 2,4-D: 0; B (control): 2,4-D: 0.5; C (control): 2,4-D: 1; D (mutant): 2,4-D: 0; E (mutant): 2,4-D: 0.5; F (mutant): 2,4-D: 1.

Table 5. Effects of 2,4-D on somatic embryo formation from embryogenic callus of

wild-type and mutant adventitious roots.

Cell line	2,4-D (mg/L)	Frequency of somatic embryo formation (%)	Number of somatic embryosper callus
	0	5.3 ± 0.73b	10.0 ± 1.2b
Wild-type	0.5	15.3 ± 1.21a	25.6 ± 2.3a
	1	2.5 ± 0.46c	4.7 ± 0.3c
	0	5.8 ± 0.28b	12.3 ± 1.9b
Mutant	0.5	14.7 ± 0.45a	23.7 ± 0.6a
	1	2.2 ± 0.27c	6.0 ± 1.4c

The data were collected after 6 weeks of culture.

The results represent the means ± SEM of values obtained from three experiments.

Different corresponding letters within a column are significant different at P<0.05 by

Duncan's multiple range test.
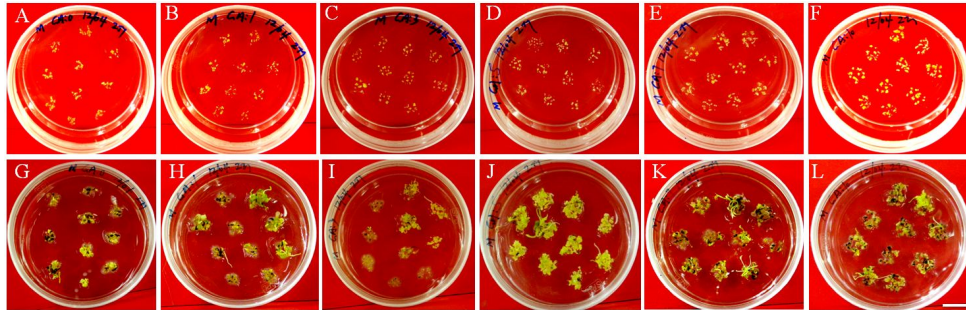
Figure 18. Effects of GA$_3$ concentration on somatic embryos germination frequency from 150 somatic embryos after 5 weeks of culture on MS medium with 3% sucrose (scale bar = 1 cm).

Table 6. Effects of GA_3 on germination of somatic embryos.

Concentration of GA_3 (mg/L)	Number of somatic embryos inoculated	Number of somatic embryos germinated	Germination frequency (%)
0	150	53 ± 6c	36 ± 4c
1	150	60 ± 9c	40 ± 6c
3	150	66 ± 10bc	45 ± 7bc
5	150	127 ± 7a	85 ± 5a
7	150	75 ± 6b	50 ± 4b
10	150	65 ± 5bc	44 ± 3bc

The data were collected after 6 weeks of culture on MS medium with 3% sucrose.

The results represent the means ± SEM of values obtained from three experiments.

Different corresponding letters within a column are significant different at $P<0.05$ by Duncan's multiple range test.
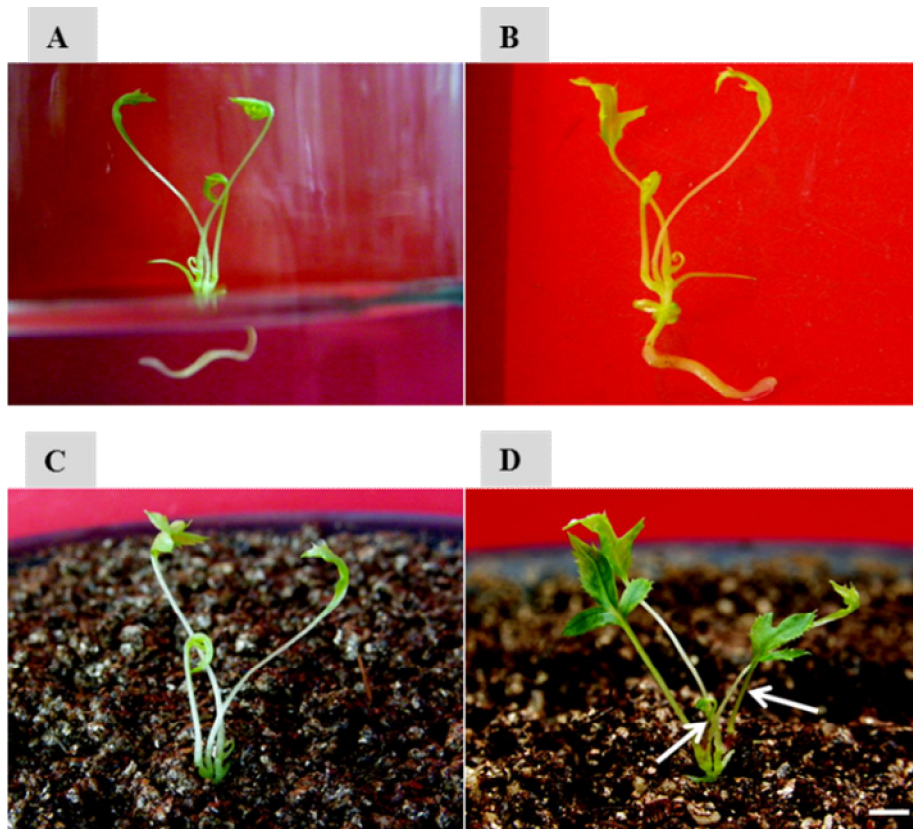
Figure 19. Development of ginseng plant and transplantation into soil. (A) Well-developed

plantlet on rooting medium, 1/3 SH basal medium supplemented with 0.25 mg/L NAA. (B)

Plant with a taproot just before transplantation into soil. (C) Regenerated plant hardened in

soil. (D) New leaves (indicated by arrows) were produced from three-month old potted plant

(scale bar = 0.8 cm).

Table 7. Comparison of rooting media for ginseng root development.

Media	No. of shoots	Root frequency (%)	No. of roots per plant	Description on root quality
1/2 MS 3% Sucrose	30	36	1.6	Grows slow, calluses
1/2 MS + 3% Sucrose + 0.5% charcoal	30	45	1.0	Grows slow
1/3 MS + 1% Sucrose + 0.25 mg/L NAA	30	58	1.0	Grows fast, thin
1/2 SH + 3% Sucrose	30	71	1.0	Grows slow
1/2 SH + 2% Sucrose + 0.5% charcoal	30	62	1.0	Grows slow, calluses
1/3 SH + 2% Sucrose + 0.5% charcoal	30	80	1.0	Grows fast, thin
1/3 SH + 1% Sucrose + 0.25 mg/L NAA	30	76	1.2	Grows fast, strong

Figure 20. Transplantation of regenerated ginseng plants into soil. (A-E) Plants derived from wild-type adventitious roots. (F-J) Plants derived from mutant adventitious roots.

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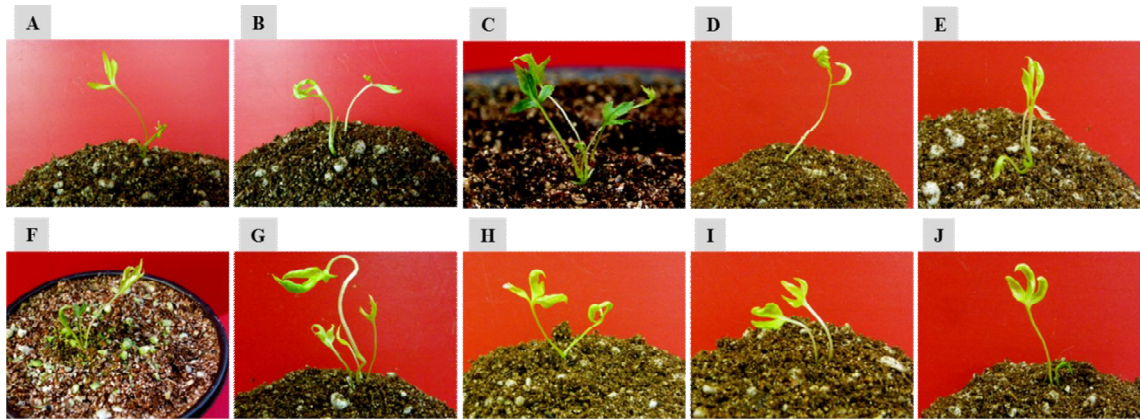Arya S, Arya ID, Eriksson T. 1993. Rapid multiplication of adventitious somatic embryos of *Panax ginseng*. *Plant Cell, Tissue and Organ Culture* 34: 157-162.

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Chapter III

Improving ginsenoside content in Korean wild ginseng by *Agrobacterium*-mediated transformation with a squalene synthase gene

INTRODUCTION

Panax ginseng Meyer is an important medicinal herb and is widely cultivated in Korea, China and Japan. The root has been used as a drug for over 2000 years in oriental countries. Its use is rapidly expanding in the Western countries as complementary and alternative medicine (Shim *et al*. 2009). Ttriterpene saponins are the major pharmacologically active components in *P. ginseng*. More than 30 types of triterpene saponins have been identified from the genus (Harrison *et al*. 1990, Nah *et al*. 1997). Triterpene saponins are produced in *P. ginseng* roots and they are referred to ginsenosides. They can be classified into three groups based on their aglycones: the protopanaxadiol-type, protopanaxatriol-type, and oleanane type saponins. The major groups of ginsenosides are Rb and Rg groups derived from the 20(S)-protopanaxadiol and 20(S)-protopanxatriol structures, respectively. Among these, ginsenosides, Rb1, Rb2, Rc, and Rd from the Rb group, and Re, Rf and Rg1 from the Rg group are the main components (Harrison *et al*. 1990; Nah *et al*. 1997). Each ginsenoside has been shown to have different pharmacological effects, including antiallergic activity, estrogen-like activity, anti-ulcer, anti-cancer, immunological activity (Bae *et al*. 2002a, Chan *et al*. 2002, Jeong *et al*. 2003, Lee *et al*. 2003 , Wang *et al*. 2000). Overproduction of triterpene saponins by metabolic engineering might be an attractive strategy to produce a better quality of pharmacologically active medicinal plants (Lee *et al*. 2004).

Attempts have been made to achieve a more rapid and increased production of the ginsenosides using other methods such as classical tissue culture system (Wu *et al*. 1999),

bioreactor culture system (Sivakumar *et al.* 2005), *Agrobacterium*-mediated transformation system (Yoshikawa *et al.* 1987, Mallol *et al.* 2001, Lee *et al.* 2004), using elicitors in cell cultures (Lu *et al.* 2001, Palazon *et al.* 2003), and mutation breeding by γ-irradiation (Kim *et al.* 2009, Kim *et al.* 2013). *Agrobacterium*-mediated transformation system and γ-irradiation have been used in many other plant species and has provided a large number of variants useful for plant breeding (Subhan *et al.* 2004, Mokobia *et al.* 2006, Chung *et al.* 2006, Lee *et al.* 2004). *Agrobacterium*-mediated transformation system and γ-irradiation enhanced ginsenoside production in *P. ginseng* (Lee *et al.* 2004, Kim *et al.* 2009, Kim *et al.* 2013). Recently, I have also generated mutant cell lines by applying γ-irradiation on *P. ginseng* adventitious roots which were derived from Korean wild ginseng root (Zhang *et al.* 2011). Among the selected mutant cell lines, line 1 has showed the highest total ginsenoside content of 7 major ginsenosides (Rg1, Re, Rb1, Rb2, Rc, Rf, and Rd). The total ginsenoside content of the mutant line was 2.3 times higher than in the wild-type line (Zhang *et al.* 2011). Using γ-irradiation, I have created a useful mutant line for breeding of the ginseng plant. However, there are no reports on in vitro *Agrobacterium*-mediated plant transformation with mutant lines of *Panax ginseng* adventitious root.

Squalene synthase (SS, EC 2.5.1.21) is a membrane bound enzyme that catalyzes the first committed step in sterol and triterpenoid biosynthesis (Abe *et al.* 1993). The enzyme plays an important role in the regulation of isoprenoid biosynthesis (Wentzinger *et al.* 2002). Both phytosterols and triterpenes in plants are synthesized from the product of cyclization of 2,3-oxidosqualene catalyzed by oxidosqualene cyclases (OSCs). The SS enzyme in

controlling sterol biosynthesis has been well characterized using *SS* mutant in animals and yeast (Karst *et al.* 1977, Tozawa *et al.* 1999). In plants, the gene has been isolated from species such as *Lotus japonicus*, *Zea mays*, etc. (Akamine *et al.* 2003, Lee *et al.* 2002). Enhanced expression of SS genes in plants such as *Panax ginseng* (Lee *et al.* 2004) and *Eleutherococcus senticosus* (Seo *et al.* 2005) resulted in increased levels of phytosterol and triterpene accumulation, thus depicting the important regulatory role of SS. Kim et al. (2011) studied that an increased expression of *SS* gene in *Bupleurum falcatum* resulted in an increased mRNA accumulation of the downstream genes of the pathway and enhanced production of phytosterol and saikosaponins in the plant.

At present no information is available on the *Agrobacterium* mediated transformation of mutant adventitious root line that has been selected from γ-irradiated *P. ginseng* adventitious roots. In this chapter, I aimed to investigate the roles of *PgSS2* on the biosyntheses of triterpenoids by using mutant cell lines of *Panax ginseng* adventitious roots through the *Agrobacterium* mediated transformation.

MATERIALS AND METHODS

Plant materials

Adventitious roots derived from Korean wild ginseng were provided by Sunchon National University. The adventitious roots were generated as described previously (Sivakumar *et al.* 2005, Yu *et al.* 2002, Kim *et al.* 2003) and have been maintained in my laboratory for over 10 years. A mutant adventitious root line has been generated from the wild type adventitious roots by γ-irradiation (Zhang *et al.* 2011). For embryogenic callus induction, mutant adventitious roots were sectioned into 10 mm in length and were placed on MS solid medium supplemented with 0.5 mg/L 2, 4-D, 0.3 mg/L kinetin and 3% sucrose. The media were solidified with 0.3% Gelite. All media were adjusted to pH 5.8 before autoclaving. Callus formation was observed after 4 weeks of culture. The induced callus was subcultured at 3 week intervals on the same medium for induction of embryogenic callus and maintenance. The embryogenic callus were used for *Agrobacterium* mediated transformation.

For extraction of total RNA and genomic DNA, adventitious roots of *Panax ginseng* were harvested after one month of growing. The transgenic *Panax ginseng* adventitious roots were collected. The tissue samples were immediately frozen in liquid nitrogen and stored at -80℃ until use.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from adventitious roots of *Panax ginseng* using Trizol (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instructions (Figure 21). The purity and concentration of RNA was checked on UV-1800 (SHIMADZU). Ideally 1 µg of this extracted RNA was directly reverse transcribed with dNTPs using M-MLV reverse transcriptase and an oligo (dT) primer in a total volume of 13.5 µL for 1 h at 42℃. The resulting cDNA mixture was directly used as a template for PCR amplification.

Homogenization in trizol reagent

Vortexing and incubation for 10 min at room temperature
Centrifugation at 13,000 rpm, 4 ℃ for 10 min

Phase separation using chloroform

Vortexing and incubation for 2 min at room temperature
Centrifugation at 13,000 rpm, 4 ℃ for 15 min

Precipitation using isopropanol and *high salt precipitation solution

*0.8 M sodium citrate, 1.2 M NaCl
Inverting and incubation at -20 ℃ for 1 h
Centrifugation at 13,000 rpm, 4 ℃ for 10 min

Wash using 70 % ethanol and *dry

Centrifugation at 13,000 rpm, 4 ℃ for 10 min
*Do not dry RNA pellets, completely.
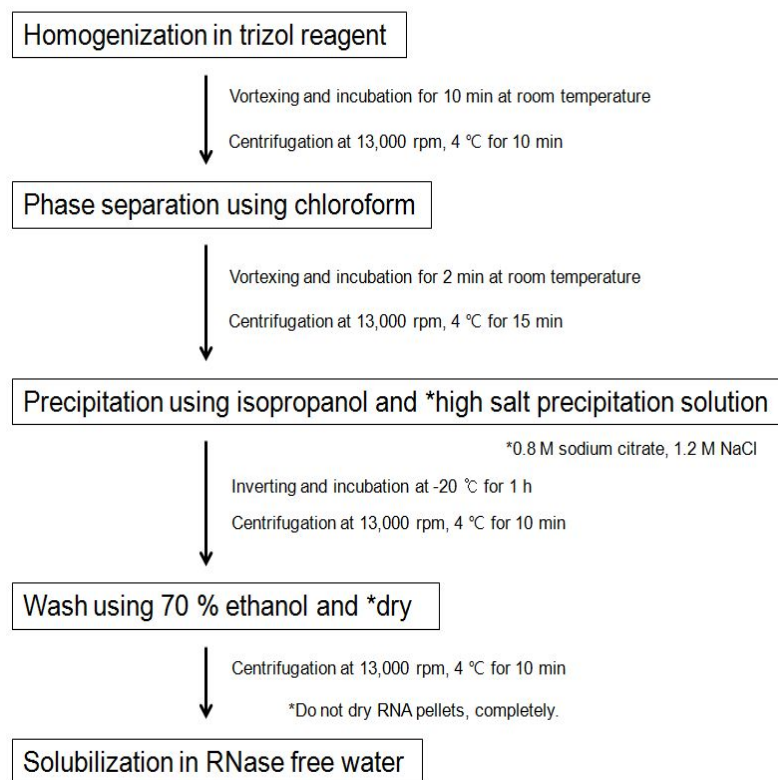
Solubilization in RNase free water

Figure 21. Total RNA extraction. Total RNA was isolated from adventitious roots of *Panax ginseng* using the trizol reagent and was dissolved in RNase free water treated with DEPC (Diethylpyrocarbonate).

TA Cloning of squalene synthase gene

Based on the nucleotide sequencing (GQ468527) from reported squalene synthase of *Panax ginseng*, specific primers were designed from synthesized for the PCR amplification of the corresponding *Panax ginseng* cDNA. The forward (SSF1) and reverse (SSR1) primers were 5'- ATGGGAAGTTTGGGGGCAATTC -3' and 5'- TCACAGGCTATTTGGTAGT-3' respectively. PCR was carried out under the following conditions: 30 cycles of 95 ℃ for 1 min, 60 ℃ for 30 s and 72 ℃ for 1 min using above cDNA as a template. An amplicon of expected size was recovered, cloned into pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* Top10 competent cells. Plasmid DNA were isolated and subjected to nucleotide sequencing.

Vector construction

The entire coding region of squalene synthase (*PgSS2*) gene was amplified from *P.ginseng* cDNA by PCR with primers having *BglII* and *PmlI* restriction sites. The resulting 1248-bp PCR product was digested with *BglII* and *PmlI*, gel purified, and ligated into the same restriction sites cauliflower mosaic virus 35S promoter followed by alfalfa mosaic virus enhancer and NOS terminator, and then the expression cassette was ligated into the same restriction sites within pCAMBIA3301 (Cambia, Australia). *Bar* gene was used as a selective marker gene (Figure 22). The freeze-thaw method was used to introduce the binary vector into *Agrobacterium tumefaciens* EHA 105 (Chen *et al*. 1994).
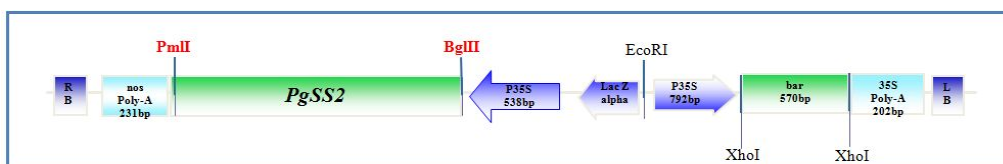
Figure 22. Construct of binary vector plasmid pCAMBIA3301-*PgSS2*. LB, left border; RB, right border; p35S, CaMV 35S promoter; 35S poly-A, CaMV 35S terminator; nos Poly-A, NOS terminator; BAR, bialaphos resistance gene.

Agrobacterium-mediated transformation protocol of *Panax ginseng*

The transformation of *Panax ginseng* was performed as reported previously (Yang *et al.* 2000) with slight modification.

(Pre-culture)   Embryogenic callus were pre-treated in MS liquid medium for 1 day.

(*Agrobacterium* culture) After an initial culture of *A. tumefaciens* in LB medium containing 75 mg/L kanamycin and 25 mg/L rifampicin for 24 h, 28°C, 200 *rpm*, the *A. tumefaciens* cells were collected using a centrifuge (3,000 *rpm*) and resuspended in MS (Murashige and Skoog 1962) liquid medium.

(Infection) Pre-cultured embryogenic callus were immersed in the suspension of *A. tumefacienes* for overnight.

(Co-culture) After overnight culture, the embryogenic callus were placed on sterilized filter paper for 10 min and transferred onto hormone-free MS medium containing 100 mg/L acetosyringone, 3% sucrose. The culture was performed at 25°C, in the dark, for 3 days.

After that, washing the embryogenic callus by using SDW until the SDW is clear and at last washing again using SDW with 1000 mg/L cefotaxime, and then the explants were

placed on sterilized filter paper for 10 min.

(Elimination) Thereafter, the callus was cultured on MS medium with 3% sucrose and 300 mg/L cefotaxime for 3 weeks.

And then the callus was transferred to selection MS medium with 3 g/L Gelite, 0.5 mg/L 2,4-D, 0.3 mg/L kinetin , 300 mg/L cefotaxime and 3 mg/L phosphinothricin (PPT).

When the callus had survived and formed embryos on the selection medium, the callus was transferred to shoot induction medium.

(Shoot induction) Selected embryos were detached and transferred to MS medium (Murashige and Skoog 1962) supplemented with 5 mg/L GA_3 and 3 mg/L PPT.

(Root induction) Then, well developed shoots were transferred onto 1/3 strength SH medium with 1% sucrose and 0.25 mg/L NAA.

Media used in the *Panax ginseng* transformation system was summarized in Table 8.

Table 8. Media used in the *Panax ginseng* transformation system.

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 Gelite, 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 Gelite, pH 5.8.
Somatic embryos induction	MS medium, 30 g/L sucrose, 0.5 mg/L 2,4-D, 3 g/L Gelite, pH 5.8.
Agrobacterium culture	Yep medium, 75 mg/L kanamycin, 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 Gelite, 100 mg/L acetosyringone, pH 5.8.
Elimination	MS medium, 30 g/L sucrose, 3 g/L Gelite, 3 00 mg/L cefotaxime, pH 5.8.
Selection	MS medium, 30 g/L sucrose, 3 g/L Gelite, 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 Gelite, 300 mg/L cefotaxime, 3 mg/L PPT, 5 mg/L GA$_3$, pH 5.8.
Root induction	1/3 SH medium, 10 g/L sucrose, 3 g/L Gelite, 300 mg/L cefotaxime, 0.25 mg/L NAA and 3 mg/L PPT, pH 5.8.

Adventitious root induction from transgenic *Panax ginseng*

To investigate the gene expression and analysis the ginsenoside content in transgenic *Panax ginseng*, leaf and hypocotyls segments of non-transgenic and transgenic plantlets

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were cultured on the MS medium with 3% sucrose, 0.3% Gelrite and 3 mg/L indolebutyric acid (IBA). Adventitious roots developed around the excised portion of segments and subcultured onto the MS liquid medium with 2 mg/L NAA, 0.25 mg/L IAA and 3% sucrose. Cultures were conducted in a culture room and maintained in a 16/8 h (light/dark) photoperiod with white fluorescent light (30 μmol m^{-2} s^{-1}) at $23 \pm 2\,°C$.

Molecular characterization of transgenic plants

Genomic DNA was isolated from adventitious roots of transgenic and wild-type plants according to CATB method (Saghai-Maroof, 1984) (Figure 23).
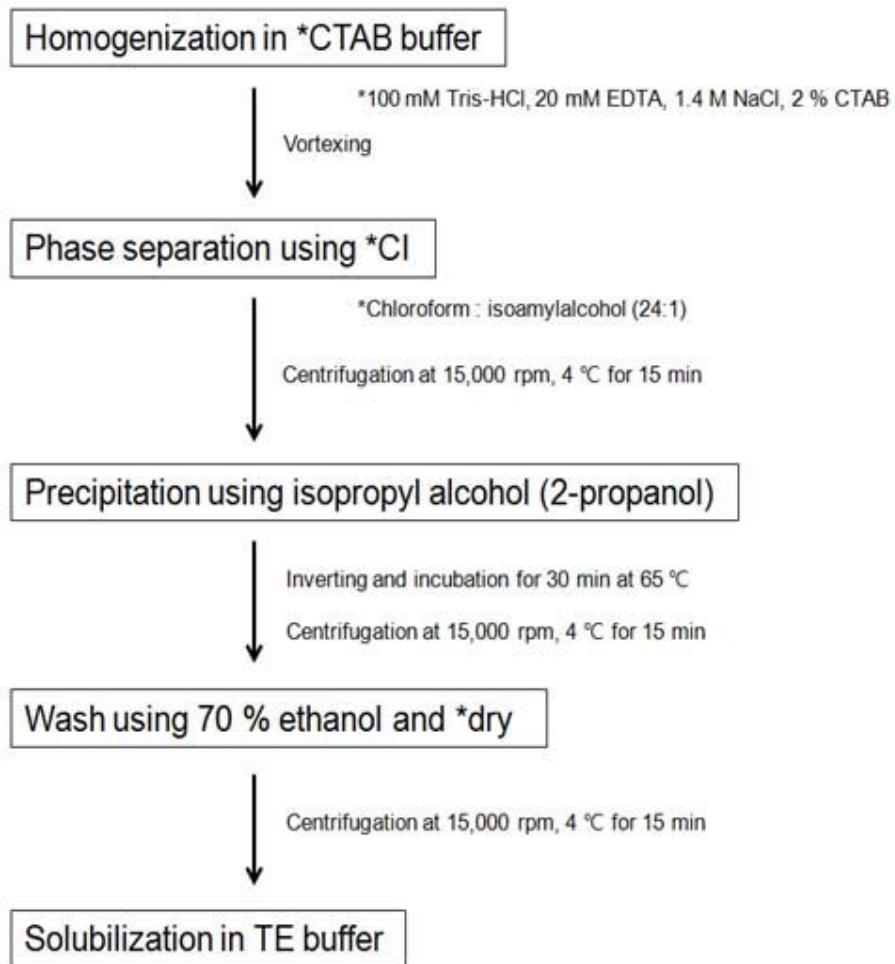
Figure 23. Extraction of genomic DNA. DNA from adventitious root of *Panax ginseng* extracted by CTAB method.

To confirm the transgene integration in transgenic plants, 10 μg of genomic DNA was digested with *BglII* (TaKaRa, Japan), which cleaves only once outside the *PgSS2* gene. The digoxigenin-labeled *SS* (340bp) and *bar* (443bp) gene specific probes were generated using a PCR DIG probe synthesis kit, according to the manufacturer's protocol (DIG System, Roche, Germany). The digested genomic DNA was fractionated on a 0.8% (w/v) agarose gel, blotted onto nylon membranes (Hybond N+, Amersham, Little Chalfont, UK), and crosslinked. The membranes were hybridized with a Dig-labeled *SS* or *bar* DNA fragment as a probe. The membranes were hybridization was carried out at 45℃ overnight in a High-SDS buffer containing 50% formamide, 5xSSC, 50 mM sodium phosphate (pH 7.0), 2% blocking reagent, and 0.1% N-lauroylsarcosine. The blots were washed twice with the 2xSSC, 0.1% SDS for 10 min at room temperature, followed by 0.1xSSC, 0.1% SDS for 15 min at 65℃. Hybridization signals were detected by chemiluminescence (CDS-star, Amersham, Little Chalfont, UK) using an alkaline phosphatase-conjugated antidigoxigenin antibody (Anti-Digoxigenin-AP Fab fragments, Roche, Mannheim, Germany), according to the manufacturer's protocol. The membrane was exposed to an LAS4000 luminescent image analyzer (Fujifilm, Tokyo, Japan) for about 1 hour (Figure 24).
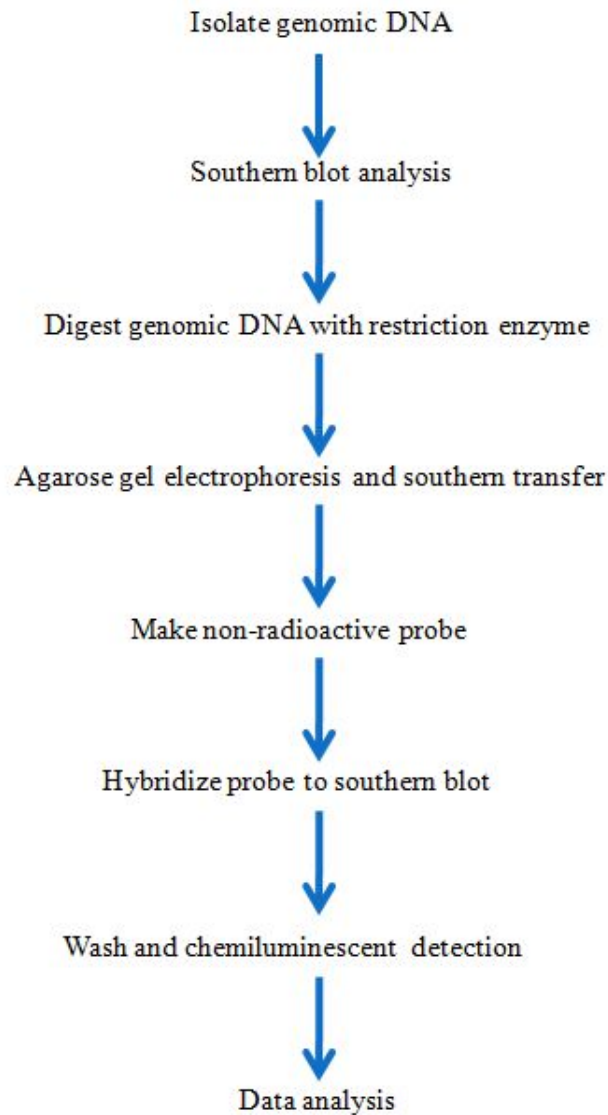
Figure 24. Scheme of Southern hybridization.

RNA from wild-type and transgenic adventitious roots were isolated with a Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers used for amplifying the *bar* gene were 5′-GCG TGA CCT ATT GCA TCT CC-3′ and 5′-TTC TAC ACA GCC ATC GGT CC-3′, and the primers for *PgSS2* gene were 5′-ATG GGA AGT TTG GGG GCA ATT CT-′3 and 5′-GTT CTC ACT GTT TGT TCA GTA GTA GGT T-′3. The PCR mixture was incubated in a DNA thermal cycler under the following

conditions: one cycle of 95℃ for 5 min, followed by 30 cycles of 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 1 min, with a final 10-min extension at 72℃. Set of primers were summarized in Table 9.

Table 9. Set of primers used for *PgSS2* gene cloning and PCR.

Set	PCR types	Genes	Oligo sequences (5` -> 3`)
1	TA Cloning	SS	Forward: ATGGGAAGTTTGGGGGCAATTC
			Reverse: TCACAGGCTATTTGGTAG
2	Vector construction	SS	Forward: AGATCTATGGGAAGTTTGGGGGCAATTC
			Reverse: CACGTGTCACAGGCTATTTGGTAG
3	RT-PCR	SS	Forward: AAGGCAGTGCAGTGCCTGAAT
			Reverse: GAATTGCTTCTAGCCTACTCAGAG
4	Probe	Bar	Forward: AAGTCCAGCTGCCAGAAACCCAC
			Reverse: GTCTGCACCATCGTCAACCACTA
5	Probe	SS	Forward: AAGGCAGTGCAGTGCCTGAAT
			Reverse: GAATTGCTTCTAGCCTACTCAGAG

Extraction of crude saponin

Extraction and determination of ginsenosides were carried out the method of Kwon *et al*. (2003) with slight modification. Ultrasonic-assisted extraction was performed with an ultrasonic water bath (Branson ultrasonics, USA). The output power is 117 volts and the frequency is 50/60Hz. Sample powder (0.5 g) was placed into a 100 mL conical flask, and then 30 mL of 80% (v/v) methanol–water were added. Then the flask was sonicated for 1 h in an ultrasonic water bath. The resultant extract was evaporated using a rotary evaporator under vacuum at 55℃. The evaporated residue (total extract yield) was dissolved in 20 mL

of distilled water and washed twice with 20 mL of diethyl ether to remove the fat contents using a separatory funnel. The aqueous layer was extracted four times with 20 mL of water-saturated n-butanol. The butanol solution was washed twice with 30 mL of distilled water to remove the impurities, thereby obtaining crude saponins. The remaining butanolic solution was transferred to the tarred round bottom flask for the evaporation using a rotary evaporator under vacuum at 55℃.

Determination of ginsenoside contents by HPLC

The HPLC conditions for ginsenoside assay was slightly modified the previous report (Park *et al.* 2007). Quantitative determinations were achieved by HPLC using a Capcell-pak C18 MG (4.6 × 250 mm) column (Shiseido, Japan), Waters 2998 Photodiode Array Detector, Waters 2690 Separations Module and Empower Program.

The solvents used were of HPLC grade. The water used was treated with a Milli-Q water purification system (Millipore, USA). Ginsenosides Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd standards were purchased from BTGin Co., Ltd (Daejeon, Korea). The HPLC conditions for ginsenoside isolation were as follows: mobile phase, water and acetonitrile; gradient elution, the eluents being 0 to 22 min, 18% acetonitrile; 22 to 32 min, 30% to 45%; 32 to 50 min, 45% to 50%; 50 to 55 min, 50% to 18%; 55 to 60 min 18%; flow rate, 1 mL/min; column temperature, 35°C; detector wavelength, 203 nm; injection volume, 10 μL.

Stock solutions for the 8 ginsenosides were prepared separately in 100% MeOH. Working solutions were prepared in methanol by mixing known amount of all the ginsenosides

together. 5 concentrations were made for standard curves, each concentration was 60, 120, 240, 320, 480 ppm. Ginsenosides were detected at a wavelength of 203 nm with the peak areas corresponding to ginsenosides from the samples matching retention times as authentic ginsenoside standards.

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:

$$GC(mg \quad g^{-1}) = \frac{SGC(fromHPLC)(mg \quad g^{-1}) \quad x \quad SV(L)}{AR(g)}$$

(GC: ginsenoside content; SGC: sample ginsenoside concentration from HPLC; SV: sample volume; AR: adventitious root)

RESULTS AND DISCUSSION

Isolation of *PgSS*2 and vector construction for *Panax ginseng* transformation

The cDNA of *PgSS2* gene was 1248-bp long and carried an open reading frame of 416 amino acids. The amino acid sequence of the *PgSS2* gene showed complete identity to previously registered squalene synthase (ACV88718). To further identify the function of *PgSS2*, functional expression of the gene was attempted. *PgSS2* cDNA was inserted into the vector pCAMBIA3301 and expressed under the control of the 35S promoter in *Panax ginseng* (Figure 25).

Establishment of an effective in vitro transformation system

Embryogenic callus were selected from mutant adventitious root line (MCL1) that recently reported by Zhang *et al*. (2014) and were used for *Agrobacterium* infection (Figure 26A, B). The genetic transformation method for *Panax ginseng* in my study was slightly modified the previous report (Chen *et al*. 2002, Choi *et al*. 2001). The binary vectors, pCAMBIA3301 and pCAMBIA3301 vector with both *PgSS2* gene and the *bar* gene, were integrated into the chromosomal genome of plant cells, so that only transformed cells could survive and then further differentiate in differentiation medium supplemented with 3 mg/L phosphinothricin (PPT). Within 2 weeks of co-culture (Figure 26B), the infected callus produced somatic embryos on embryo selection medium (Figure 26C, D). The selected somatic embryos were transferred into shoot selection medium with 3 mg/L PPT (Figure 26E), and then, the selected shoots were transferred into root induction medium with 3 mg/L

PPT (Figure 26F). To produce independent transgenic *Panax ginseng* lines, somatic embryos were detached from the transgenic embryogenic callus and cultured on shoot induction medium as described by Choi *et al*. (2001) and Lee *et al*. (2004). Ten PPT-resistant plant lines were generated on selection medium (Figure 27) and the integration of the transgene in these plants was confirmed by PAT test strip (Figure 28).

Production of transgenic adventitious roots

To investigate the transgene integration and the ginsenoside content in transgenic *Panax ginseng*, leaf and hypocotyl segments of non-transgenic and transgenic plantlets were cultured onto MS medium with 3% sucrose, 0.3% Gelrite and 3 mg/L indolebutyric acid (IBA), as previously reported by Lee *et al*. (2004). Transgenic adventitious roots were formed directly on the surfaces of leaf and hypocotyl segments after four weeks of culture (Figure 29). The induced transgenic adventitious roots were proliferated in flask culture (Figure 30).

Molecular analysis of putative transgenic adventitious root

The expression of *PgSS2* and *bar* genes in transgenic adventitious roots was confirmed by RT-PCR. The PCR analysis showed clear bands for *SS* gene (340 bp) and *bar* gene (443 bp) but no bands in the wild-type (Figure 31). Ten selected transgenic lines were analyzed by Southern hybridization (Figure 32). In case of SS probe, hybridization signal was detected in non-transgenic control plants and transgenic plants, because SS gene is endogenous gene and

might be consisted with a multicopy gene and/or large gene with several introns (Lee *et al.* 2004). In case of bar probe, all of the 10 transgenic lines showed the hybridization signal but not in the non-transgenic control plants. The genomes of two of the transgenic plants carried one copy of the *bar* gene, and the other plants contained at least two copies of the gene. These results showed that the foreign genes are successfully integrated into the *P. ginseng* genome. In dicotyledonous plants, T-DNA integrates either at one locus or at several independent loci. In addition, multiple T-DNA copies frequently are formed at one locus, in either direct or inverted repeats (Kim *et al.* 2003).

Accumulation of ginsenoside content (Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd) in *Panax ginseng* overexpressing squalene synthase (*PgSS2*) gene

The total ginsenoside contents in adventitious roots of transgenic *P. ginseng* were analyzed by HPLC. The chromatogram for 8 major ginsenosides among each transgenic cell lines was shown in Figure 33-42. The total contents of the 8 ginsenoside types were significantly enriched in the 5 transgenic lines compared to the mutant control (MCL1). The line SS 1 and 8 showed a 1.6-fold increase than the MCL1 (Figure 34; Figure 41). The line SS 2 showed a 1.5-fold increase than the MCL1 (Figure 35), and the line SS 5, SS 7 showed a 1.3-fold increase than the MCL1. However, in the line SS 4 and 5, the total contents of ginsenosides were less than that of the MCL1. The total contents of the 8 ginsenoside types (Rg1, Re, Rf, Rh1, Rb1, Rc, Rb2 and Rd) obtained from the mutant control (MCL1), empty vector and the SS lines were 6.14 mg g^{-1}, 6.07 mg g^{-1}, 9.82 mg g^{-1}, 9.06 mg g^{-1}, 5.01 mg g^{-1},

4.37 mg g^{-1}, 8.22 mg g^{-1}, 6.46 mg g^{-1}, 8.05 mg g^{-1}, 9.75 mg g^{-1}, 4.56 mg g^{-1}, respectively. Overall, the highest total ginsenoside content was obtained in line SS 1 and 8 (Figure 43).

This study demonstrated that it is available to induce the hyperaccumulation of triterpene saponins by molecular breeding. The *Agrobacterium*-mediated transformation of *SS* gene in *Panax ginseng* showed that the total ginsenoside content of the transgenic plants was increased a 2-3 fold compared to the control (Lee *et al.* 2004). *SS* gene plays an important regulatory role in the biosynthesis of ciwujianosides in *E. senticosus* (Seo *et al.* 2005). Overexpression of *PgSS2* gene by genetic transformation should be useful for the efficient production of pharmacologically important ginsenosides in *Panax ginseng*.

CONCLUSIONS

The production of transgenic ginseng plants through somatic embryogenesis by using adventitious root explants can be applied to genetic transformation of *Panax ginseng*, although long time (about 8 months) is required for the production of transgenic plantlets from the explants. Improving the yield and quality of medicinal plants and crops that are the source of triterpene saponins has become an important issue. In this study, I established the *Agrobacterium*-mediated transformation of *PgSS2* gene into *Panax ginseng* by using mutant adventitious roots as explants. Overexpression of *PgSS2* gene should be useful for the mass production of ginsenosides of triterpene saponins in *Panax ginseng* by genetic transformation. Metabolic engineering of *Panax ginseng* through the transgenic adventitious root culture can be an important technique to upgrade medicinal value of roots and efficient production of secondary metabolites from roots. This transformation method may facilitate the improvement of *Panax ginseng* in terms of the accumulation levels of ginsenoside. The combination of mutation technique by γ-irradiation, plant regeneration by tissue cultures and genetic transformation may be an effective way to develop new cultivars of *Panax ginseng*.
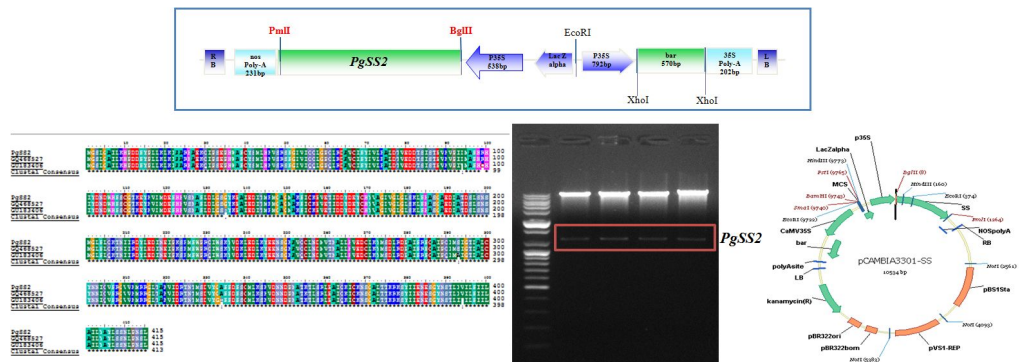
Figure 25. TA cloning of squalene synthase gene from *Panax ginseng* adventitious root and T-DNA region of binary vector (pCAMBIA3301-*PgSS2*) construct used for *Agrobacterium*-mediated transformation.
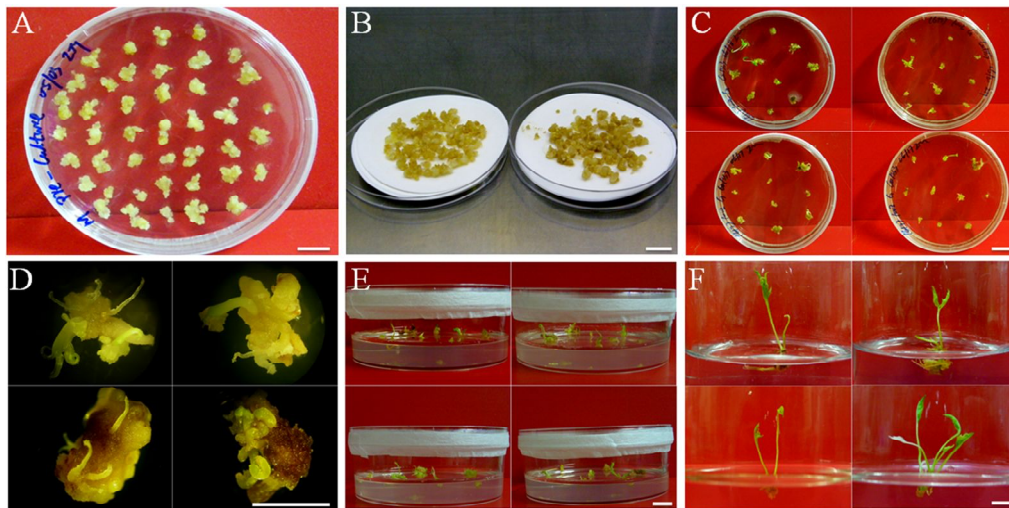
Figure 26. Transformation procedure of *Panax ginseng* adventitious root. (A) Embryogenic callus induced from mutant adventitious roots; (B) Infected and co-cultivated embryogenic callus; (C) Embryo induction from embryogenic callus; (D, E) Germination and shoot elongation (D, scale bar = 2mm); (F) Root induction (scale bar = 1cm).
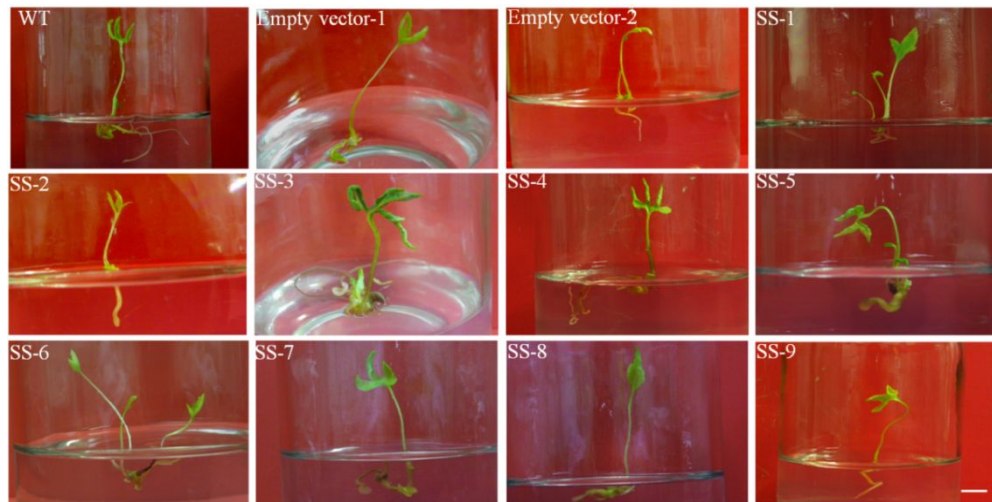
Figure 27. Phosphinothricin-resistant plant lines generated from *Panax ginseng* adventitious roots. WT, non-transgenic control plant; Empty vector , transgenic plants with empty vector; SS1-SS9, transgenic plant lines (Scale bar = 1cm).
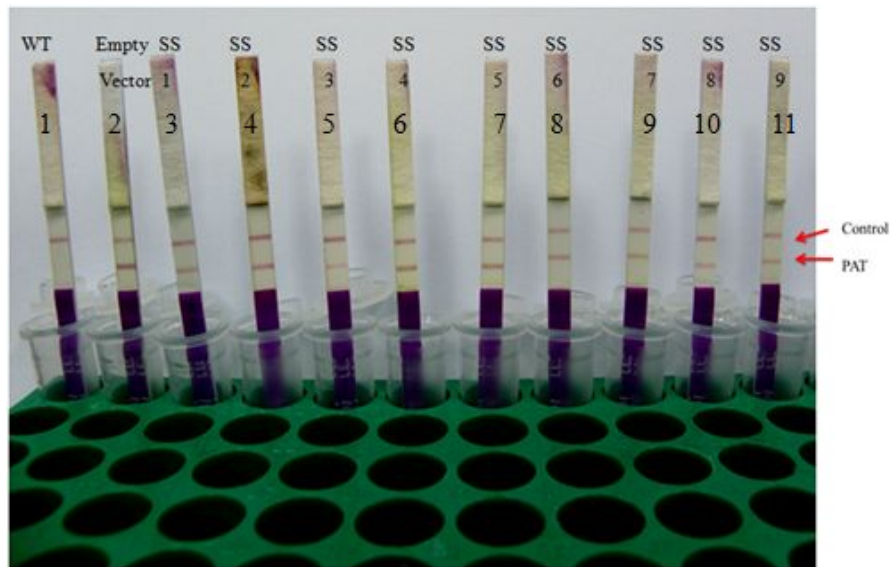
Figure 28. PAT test strip analysis of transformants. WT, non-transgenic control plant; Empty

vector , transgenic plants with empty vector; SS1-SS9, transgenic plant lines.
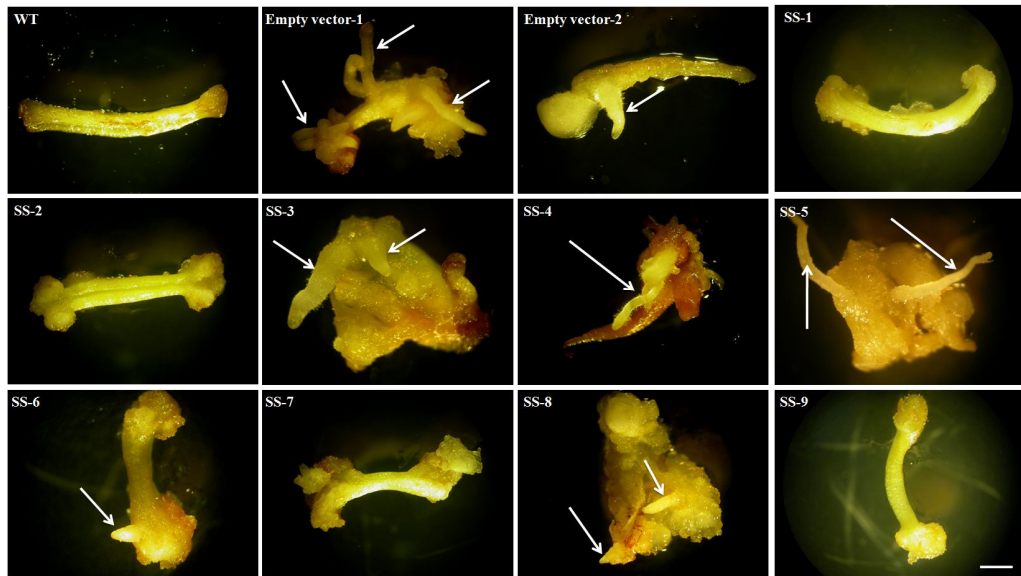
Figure 29. Adventitious root induction from transgenic *Panax ginseng* shoot on MS medium supplemented with 3 mg/L IBA. WT, non-transgenic control plant; Empty vector , transgenic plants with empty vector; SS1-SS9, transgenic plant lines.
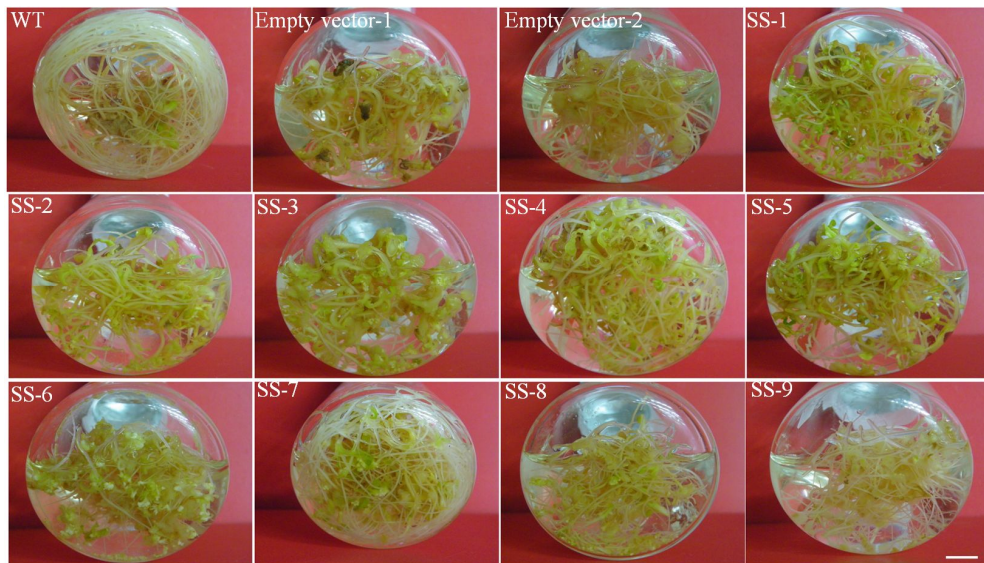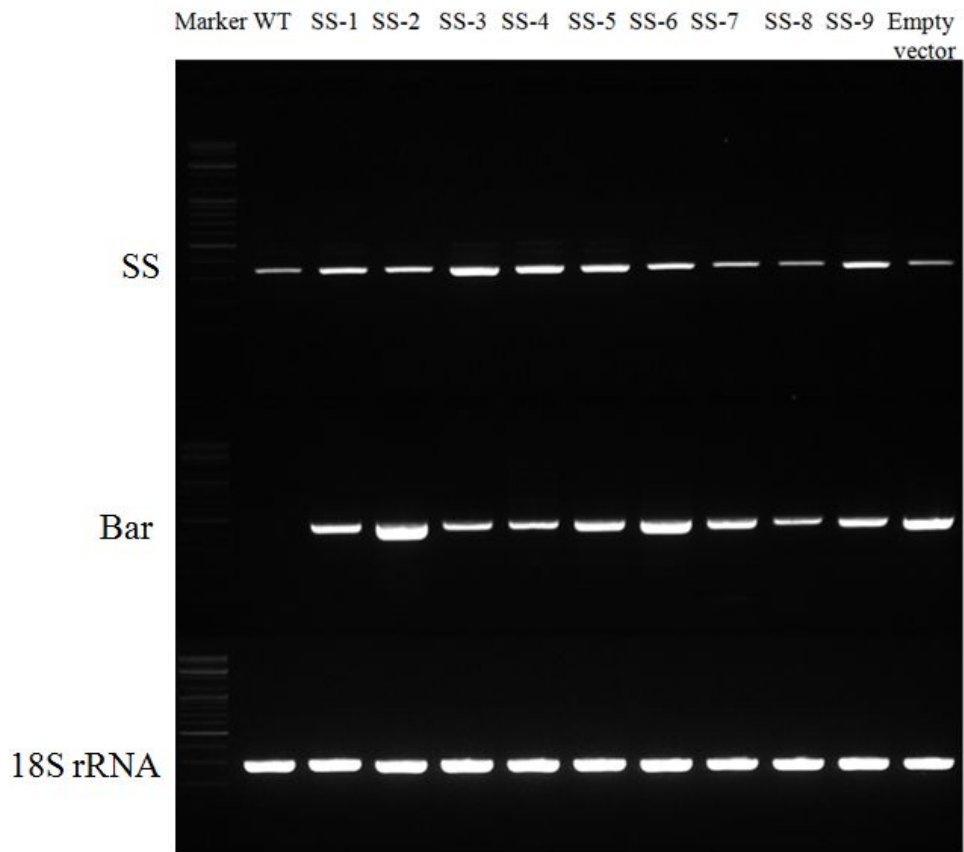
Figure 30. Adventitious root proliferation from transgenic *Panax ginseng* shoot on MS medium supplemented with 2 mg/L NAA and 0.25 mg/L IAA. WT, non-transgenic control plant; Empty vector , transgenic plants with empty vector; SS1-SS9, transgenic plant lines.

Marker WT SS-1 SS-2 SS-3 SS-4 SS-5 SS-6 SS-7 SS-8 SS-9 Empty vector

SS

Bar

18S rRNA

Figure 31. Transgene expression in wild type and transgenic plant lines of *P. ginseng*. WT, non-transgenic control plant; Empty vector , transgenic plants with empty vector; SS1-SS9, transgenic plant lines.
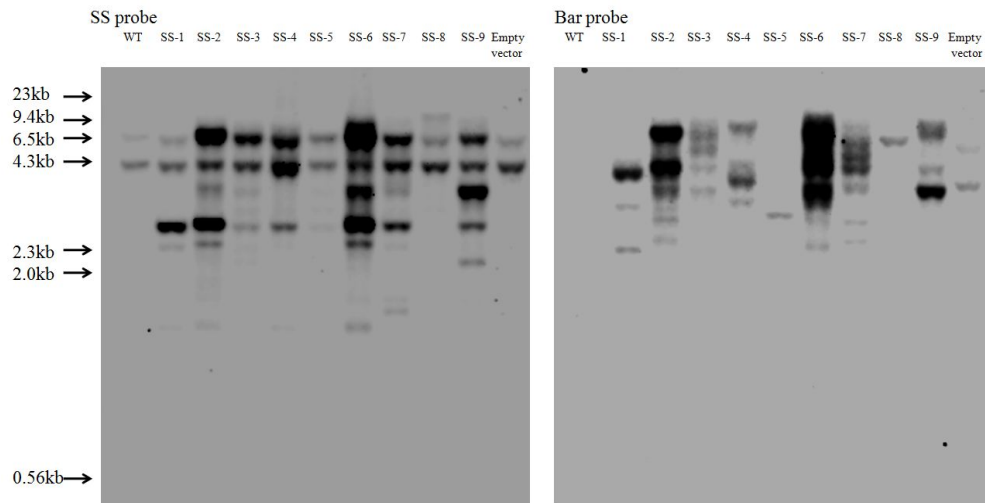
Figure 32. Southern blot analysis of transgenic *P. ginseng*. Genomic DNAs extracted from ten different transgenic *P. ginseng* lines. Genomic DNA (10 μg) was digested with the restriction enzymes (*BglII*), separated on 1% agarose gel, transferred to nylon membrane, 0.3 kb SS gene and 0.4 kb bar gene fragment was used as a probe for Southern blot hybridization. WT, non-transgenic control plant; Empty vector , transgenic plants with empty vector; SS1-SS9, transgenic plant lines.
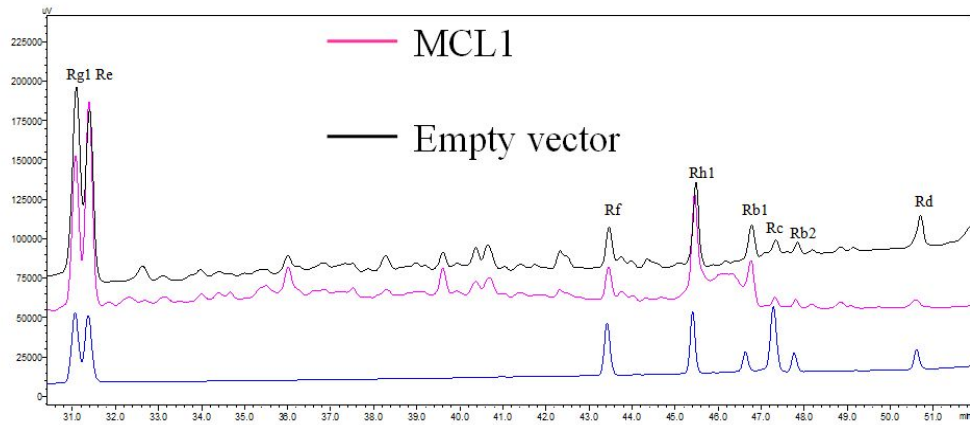
Figure 33. Typical chromatograms and ginsenoside content obtained from the standard, mutant control (MCL1) and transgenic *Panax ginseng* with empty vector.
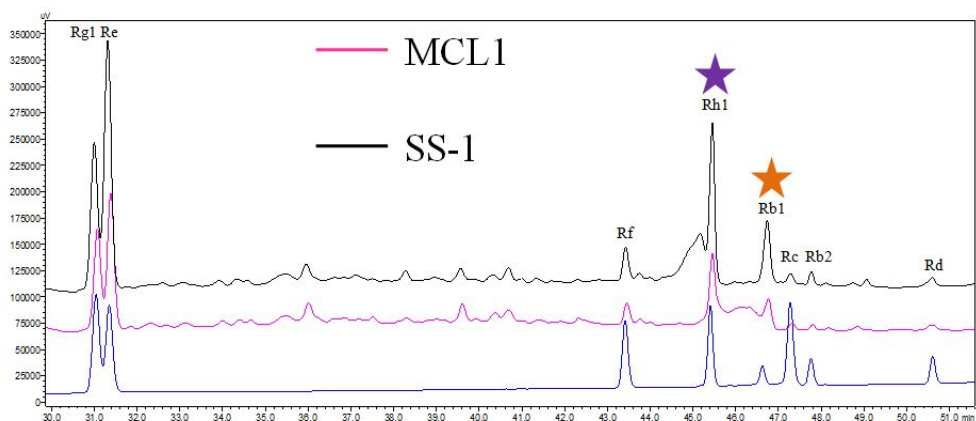
Figure 34. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-1. The ginsenosides were extracted from adventitious roots cultured in the flask.
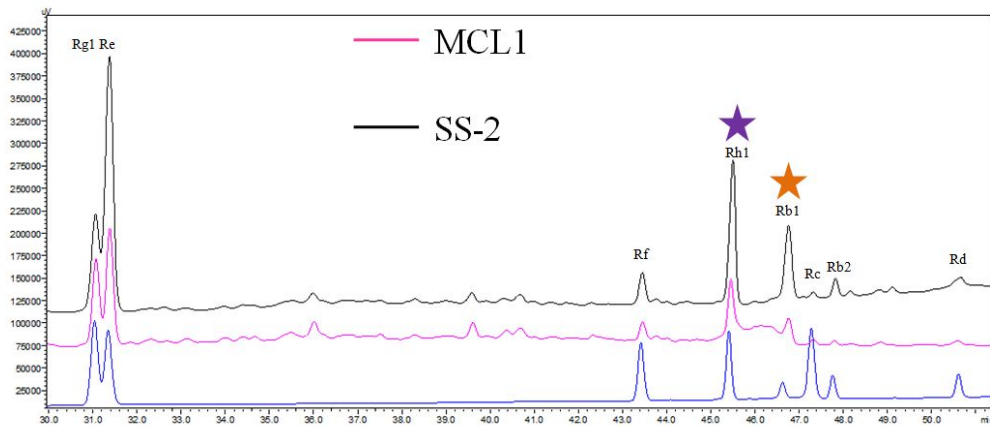
Figure 35. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-2. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 36. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-3. The ginsenosides were extracted from adventitious roots cultured in the flask.
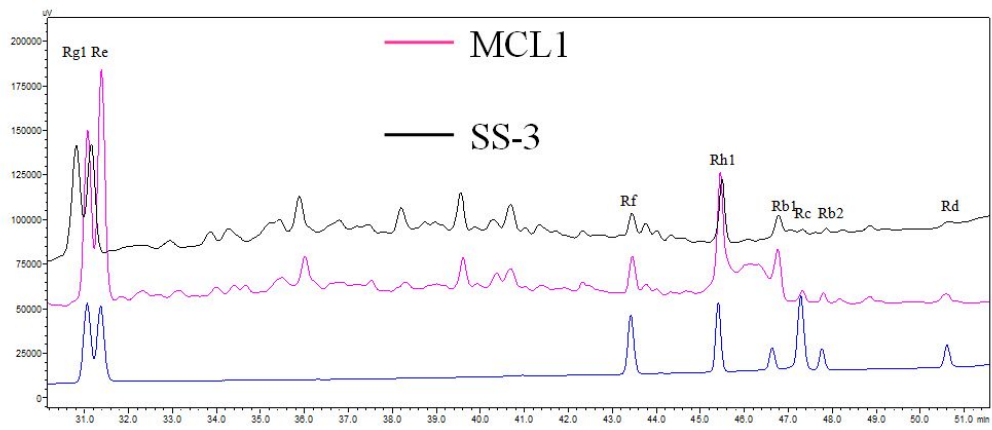
Figure 37. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-4. The ginsenosides were extracted from adventitious roots cultured in the flask.
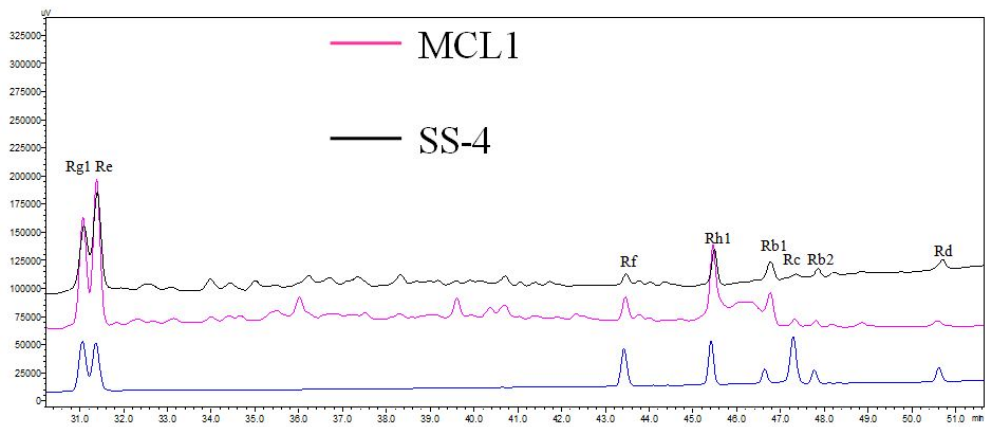
Figure 38. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-5. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 39. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-6. The ginsenosides were extracted from adventitious roots cultured in the flask.
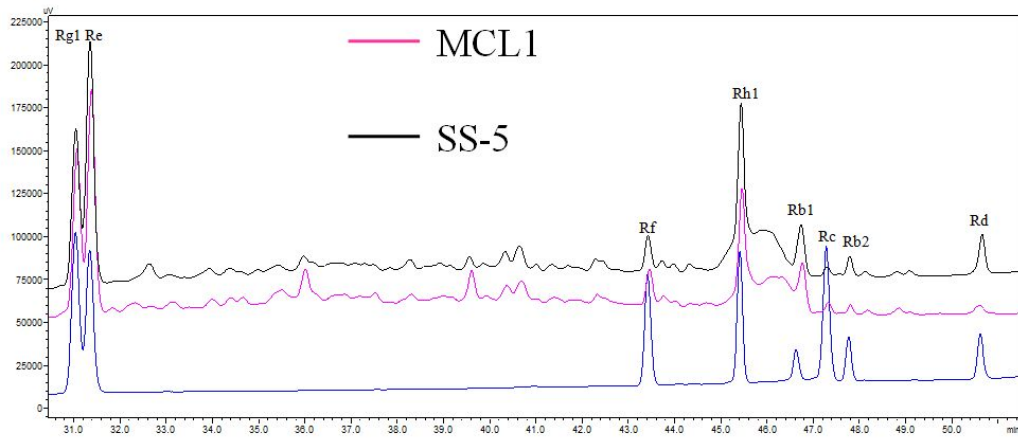
Figure 40. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-7. The ginsenosides were extracted from adventitious roots cultured in the flask.

Figure 41. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-8. The ginsenosides were extracted from adventitious roots cultured in the flask.
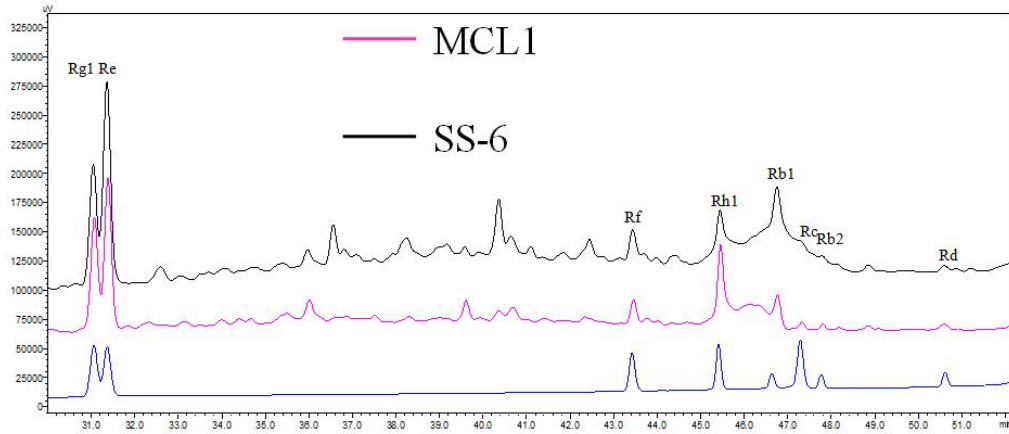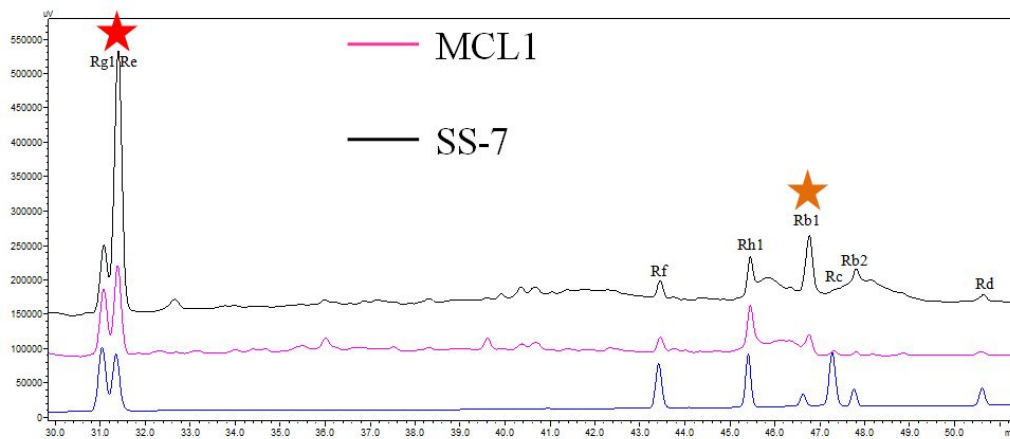
Figure 42. Typical chromatograms and ginsenoside content obtained from the standard solution, mutant control (MCL1) and transgenic *Panax ginseng* line SS-9. The ginsenosides were extracted from adventitious roots cultured in the flask.

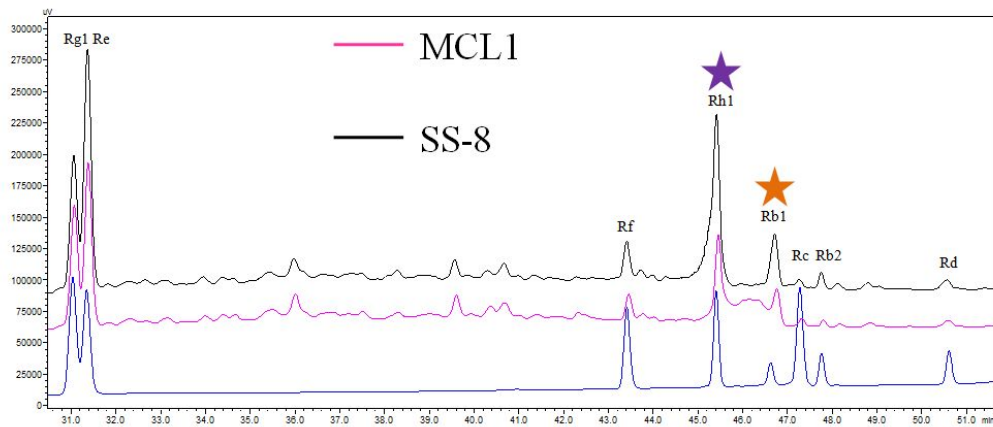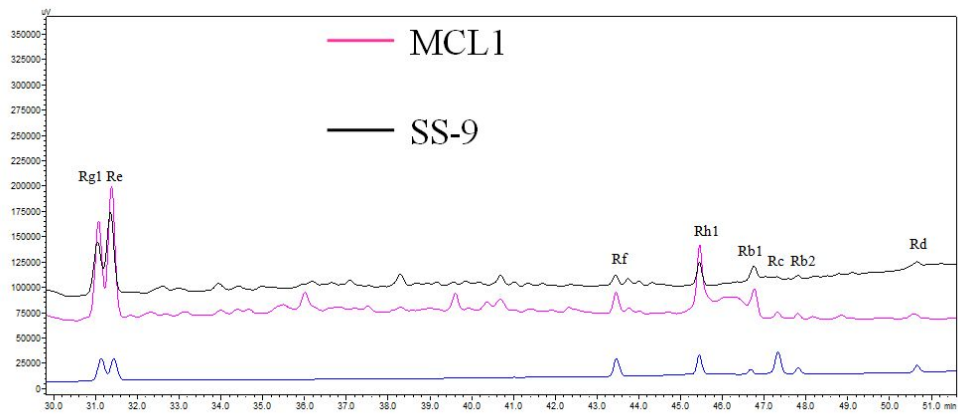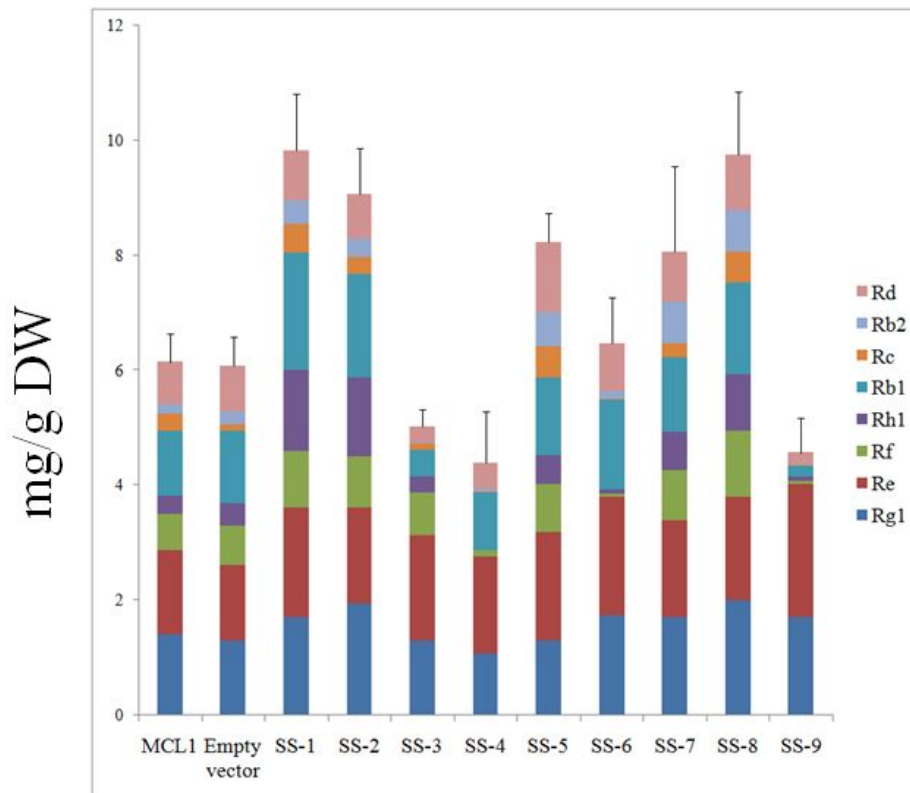Figure 43. Total ginsenoside content of the mutant control (MCL1) and transgenic lines of

Panax ginseng. MCL1: non-transformed mutant control, Empty vector: transgenic *Panax ginseng* with empty vector, SS-1 – SS-9: transgenic *Panax ginseng* lines.

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PUBLICATIONS

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<u>Jun-Ying Zhang,</u>[1] Tae-Woong Bae,[2] Kyung-Hwan Boo,[3] Hyeon-Jin Sun,[2] In-Ja Song,[1,2] Chi-Hoa Pham,[4] Markkandan Ganesan,[1,2] Dae-Hwa Yang,[1] Hong-Gyu Kang,[2] Suk-Min Ko,[2] Key-Zung Riu,[1,2] Pyung-Ok Lim,[2,5] and Hyo Yeon Lee[1,2,*]. "Ginsenoside Production and Morphological Characterization of Wild Ginseng (*Panax ginseng* Meyer) Mutant Lines Induced by γ-irradiation (^{60}Co) of Adventitious Roots". *Journal of Ginseng Research* 2011 September; 35(3): 283–293.

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