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THESIS FOR DEGREE OF MASTER OF SCIENCE

Development of tissue culture system of interspecies hybrid *Panax ginseng* x *Panax quinquefolius*

인삼과 미국삼간의 교잡종 조직배양시스템 개발

BY

MIN-JUNG KIM

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MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

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Development of tissue culture system of interspecies hybrid *Panax ginseng* x *Panax quinquefolius*

MIN-JUNG KIM

ABSTRACT

Panax ginseng C. A. Meyer (PG) and *Panax quinquefolius* L. (PQ) have been widely used as medicine and functional food material. They are closely related allotetraploids with slightly different ginsenoside profiles. Interspecies hybrid (Hybrid) between PG and PQ shows inter-species hybrid vigor for growth rate and ginsenoside contents. However, the interspecies hybrid is sterile with immature pollen grains. To overcome infertility of interspecies hybrid lines, we developed multiplication tissue culture system from one immature berry of the hybrid plant. We induced various tissues such as callus, adventitious root, somatic embryo and hairy root by providing optimal composition of plant growth regulators (PGRs) and the medium. We successfully induced multi shoots from the somatic embryo and complete plantlets. The Hybrid showed more vigorous growth of adventitious root culture compare to its parental species. The fresh weight of the Hybrid was 1.58 and 2.06 times higher than PQ and PG, respectively. *Agrobacterium rhizogenes*-mediated transformation induced hairy roots of the Hybrid and it grew approximately 2.49 times faster even though without the supplementation of PGR

when it compared to the adventitious roots grown with the supplement of PGRs. We confirmed the interspecies hybrid by applications of chloroplast unique markers and nuclear SSR markers. The hybrid has 45 kinds ginsenosides which have most kinds of PG-unique and PQ-unique ginsenosides. This study will serve a platform for ginseng breeding and ginseng industry based on the inter species hybrid.

Keywords: *Panax ginseng, Panax quinquefolius,* ginsenoside, interspecies hybrid, inter-species hybrid vigor, sterile, propagate, plant growth regulators (PGRs), *Agrobacterium rhizogenes*

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ABBREVIATION

PGRs	Plant growth regulators
2,4-D	2,4-Dichlorophenoxyacetic acid
IBA	Indole-3-butyric acid
NAA	Alpha-Naphthaleneacetic acid
BA	6-Benzylaminopurine
LB	Luria broth medium
MS	Murashige and Skoog medium
SH	Schenk and Hildebrandt medium
SSR	Simple sequence repeat
dCAPS	Derived cleaved amplified polymorphic sequences
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
CDS	Chloroplast coding sequence
EtBr	Ethidium bromide
ESI	Electrospray ionization
UPLC	Ultra performance liquid chromatography
QTOF	Quadrupole time of flight
MS	Mass spectrometry
G	Ginsenoside

INTRODUCTION

Several plants in genus *Panax* (Araliaceae) are highly valued herbs and have been used as drugs for a long time [1]. Among 17 species in the *Panax* genus [2], Korean ginseng (*Panax ginseng* C. A. Meyer, PG) and American ginseng (*Panax quinquefolius* L., PQ) are allotetraploid plants (2n=4x=48) that have been mainly used as medicine and functional food material [3]. Through phylogenetic relationships using internal transcribed spacer (ITS) regions in ribosomal RNA [4], chloroplast DNA [5] and homologous genes [6], it was found that both species have diverged very recently (ca. 0.8~1.2 MYA) via migration from their common ancestor [6] and have a close phylogenetic relationship [7]. To date, *Panax ginseng* breeding had been researched consistently, and three pure lines and 12 cultivars were registered in Korea [8]. One of these cultivars, Yunpoong, exhibited characteristics of higher stem incidence, larger root thickness and higher yield when compared to other cultivars [9].

Secondary metabolites of ginseng (also called ginsenosides) were reported to have pharmacological effects such as central nervous system (CNS) promotion, wound recovering, anticancer activities and antioxidative activities [10] [11]. More than 150 ginsenosides have been identified and quantified [12]. Particularly, PG and PQ showed different ginsenoside profiles; pseudoginsenoside F_{11} was not detected in PG and ginsenoside Rf was a lot more abundant in PQ than in PG [13].

The hybrid (Hybrid) between PG cultivar Yunpoong and PQ yielded higher biomass and secondary metabolite production than its parents because of inter-

species hybrid vigor or heterosis. Such outcome intrigues the use of hybrid technology throughout fields of horticulture and agriculture. However, ginseng interspecies hybrid also casts a problem of undeveloped normal pollen grain, and they show cross sterility [14].

Ginseng grows slowly and produces their seeds after 3~4 years of growth. In the field, there are several disturbing elements for the yield and quality of ginseng: red skin disease leading to replant failure, long cultivation period, and extensive labor requirement. In order to obtain ginseng seedlings from the seeds, conventional methods in the field also require several months of labor. To reduce time and effort, many attempts have been tried to accelerate ginseng plant production by means of somatic embryogenesis and adventitious root development using tissue culture technique. These methods enabled the propagation of superior lines, the production of numerous progenies, and the decrease of inherent variability [15]. It also could overcome the infertility issue and maintain sterile interspecies hybrid lines. The application of tissue culture system for ginseng adventitious root cultures gave an effective approach in acquiring higher ginseng biomass and secondary metabolite production due to fast growth as well as stable culture system [16] [17].

The plant hormone, auxin has been known as an important plant growth regulator that affects plant cell division, morphological change and organ differentiation. During tissue culture in *Panax* species, 2,4-D (2,4-Dichlorophenoxyacetic acid, a synthetic auxin) was necessary for the initial callus formation from seed, leave or root tissues [18]. In addition, a previous study showed that IBA (Indole-3-butyric acid, a synthetic auxin) was more effective than

2,4-D regarding ginseng organ formation, including somatic embryogenesis, and induction or proliferation of ginseng adventitious root [19] . On the basis of this previous study, we have decided to use a medium containing IBA to increase the yield of adventitious root for this study.

Sequence-based DNA markers are accurate, affordable and convenient tools in species identification. Moreover, marker assisted selection was an efficient method for breeding. Two types of DNA markers, simple sequence repeat (SSR) markers and derived cleaved amplified polymorphic sequences (dCAPS) markers, were designed based on nuclear and chloroplast genome sequence variance. Each developed marker will help distinguish *Panax* species and its maternal line to create Hybrid line of ginseng [7].

Here, in order to maintain the sterile Hybrid of the representative medical plants, PG and PQ, we tried to establish suitable medium for the induction and proliferation from its flesh from one immature berry to various tissues, including somatic embryo, adventitious root, hairy root, leaf and an individual plant. We have confirmed the parents of the Hybrid as well as its maternal line through molecular markers. We also compared the ginsenoside contents of PG, PQ and their Hybrid by UPLC-QTOF/MS. We could maintain a superior Hybrid line with higher yields of root biomass and ginsenosides by propagating into various tissues. This study will play a big role in future demand of ginseng-containing products in the ginseng industry.

MATERIALS AND METHODS

Plant materials and sterilization

All plant materials were collected from National Institute of Horticulture and Herbal Science, South Korea. To produce Hybrid, PG (Yunpoong cultivar) used as maternal parent and PQ (collected from Gillin of China) used as a pollen donor. To incubate plant materials in vitro, dehisced thick seed coats were removed using forceps. Naked seeds were washed by shaking with a detergent for 30 min and rinsed five times in a water. They were immersed in 70 % EtOH for 45 sec~1 min and submerged in a 0.5~1.0 % solution of commercial bleach (Javex, containing 4.5 % NaOCl) in the clean bench. After disinfection and sterilization, they were washed five times with distilled water and dried using sterile papers.

Culture media and incubation conditions

It was important to secure sufficient quantity of callus since they are good starting points for making various tissues, including adventitious root, somatic embryo, stem, leave and plantlet. We rescued the embryos from seeds of PG and PQ as well as a flesh from one immature berry of Hybrid and placed them on MS medium [20] containing 1.0 mg L^{-1} 2,4-D in darkness till callus appeared. During the first year, as callus regenerated, the number of them continuously increased by growing and cutting to the appropriate size.

Next, proliferated calli were transferred onto medium supplemented with a diverse range of auxin concentrations with 2,4-D (1.0 and 1.5 mg L^{-1}) in combination with IBA (0, 0.5 and 1.0 mg L^{-1}) (Figure 2).

All culture media were composed of MS medium solidified with 7 % (w/v) plant agar (duchefa biochemie) supplemented with 3 % sucrose (duchefa biochemie) and plant growth regulators (duchefa biochemie) at various concentrations. After adjusting the pH of media to 5.8, they were autoclaved at 121 ℃ for 15 min at 105 kPa. Embryos and cotyledons were placed on 25 mL of MS medium with 12 calli per 90 x 20 mm Petri dish and subcultured to fresh medium every 4 weeks.

Adventitious root culture

For adventitious root culture incubation, root fragments with callus (Figure 2) were cultivated on SH medium [21] containing 3.0 mg L^{-1} IBA until they grew longer. After 6-8 weeks, only adventitious roots were separated from callus, that were then transferred to liquid SH medium with different concentrations of IBA (0, 2.0, 4.0, 6.0 mg L^{-1}), conducted three times (Figure 3). Approximately 0.4~0.7 g of them were transferred to SH medium supplemented with IBA (0, 2.0, 4.0, 6.0 mg L^{-1}) per 100 mL of glass erlenmeyer flask with 50 mL of culture medium for 4 weeks in a shaking incubator (KH-201SL, Hanbaek Scientific Co, Korea) at 22 ℃ and 110 rpm in the dark.

Somatic embryogenesis culture

For somatic embryogenesis, We made sure of that callus induced somatic embryos on MS medium containing 1.5 mg L^{-1} 2,4-D in combination with 0.5 and 1.0 mg L^{-1} IBA (Figure 2), and transferred the somatic embryos to MS with combination of 1.0 mg L^{-1} NAA (alpha-Naphthaleneacetic acid) and 0, 0.5 and 1.0 mg L^{-1} BA (6-Benzylaminopurine). Moreover, when the somatic embryos with callus were moved to MS supplemented with 1.0 mg L^{-1} NAA in combination with 1.0 mg L^{-1} BA and 0.5 and 1.0 mg L^{-1} GA_3, lengthening of the plantlet stem was observed. The several somatic embryos per callus were divided containing 1~2 plantlets using razor blades. It would grow better than when status of multiple somatic embryos.

Hairy root culture

Agrobacterium rhizogenes R1000 strain was used in this study and the binary vector pBI121 [22] containing an *NPT Ⅱ* gene as a selection marker was transferred into *A. rhizogenes* R1000 by electroporation. Transformed bacterium were cultured on Luria broth (LB) medium containing kanamycin (50 mg L^{-1}) for 1 day in the shaking incubator at 28 ℃ and 110 rpm under the dark [23]. After the bacteria were centrifuged at 3500 rpm for 10 min, the supernatant was discarded and the pink pellet was mixed with a half MS medium. Calli of Hybrid were immersed for 10 min in the bacteria culture solution. Infected explants were transferred to sterilized filter papers (Whatman No. 2) to remove excess solution and cultured on a half MS medium for 2 days in the darkness. After 2 days, co-cultured calli and bacteria were washed several times with sterile distilled water

and dried using filter papers to remove bacteria. Calli were incubated on a half medium containing kanamycin (50 mg L^{-1}) and cefotaxime (250 mg L^{-1}) which kills excess bacteria for 8 weeks at room temperature in darkness. When the hairy roots began to grow longer than 5cm from calli, the hairy roots were separated from calli and transferred to liquid SH medium containing kanamycin (50 mg L^{-1}) and cefotaxime (250 mg L^{-1}) and cultured in a shaking incubator for 4 weeks at 28 ℃ and 110 rpm under the dark. After 4 weeks, the transgenic hairy roots were transferred to hormone-free liquid SH medium containing 3.0 mg L^{-1} IBA without kanamycin and cefotaxime. Data were collected after 4 weeks of culture.

Primers

DNA sequence of the primers sets used in this study were listed in Table 1.

PCR analysis of *NPT Ⅱ* gene (the selection marker)

PCR reactions were performed in a total volume of 25 μL containing 2 μL DNA template, 1 μL primer, 0.5 μL deoxynucleoside triphosphate (dNTP), 0.25 μL Taq DNA polymerase (Inclone biotech, Korea), 2.5 μL 10X reaction Buffer and 18.75 μL water. The quality of extracted genomic DNA was measured using a DNA engine thermal cycler (Biorad, Hercules, CA, USA). Cycling conditions were 5 min at 96 ℃ for denaturation followed by 30 cycles of 30 sec at 94 ℃, 30 sec at 55 ℃, 1 min at 72 ℃ for polymerization and 10 min at 72 ℃ for final extension. After PCR reaction, products were separated in 3 % agarose gels stained with safe gel and visualized.

DNA isolation and DNA markers

Total genomic DNA was extracted from fresh adventitious roots using modified cetyltrimethylammonium bromide protocol [24] and DNA concentrations were measured using ND-1000 (Nanodrop Technologies Inc.,Wilmington, DE, USA).

To discriminate parents (PG and PQ) of Hybrid, primers of simple sequence repeat (SSR) and derived cleaved amplified polymorphic sequence (dCAPS) markers which had been designed in our lab were used [25] [26] (Table 1). Four SSR markers were designed based on ESTs including 3~6 copies of SSR motifs from conserved genic regions. Six dCAPS markers were designed from single nucleotide polymorphism (SNP) sites in the chloroplast coding sequence (CDS).

Polymerase chain reaction (PCR) and electrophoresis

PCR reactions were performed in a total volume of 25 μL containing 2 μL DNA template, 1 μL primer, 0.5 μL deoxynucleotide triphosphate (dNTP), 0.25 μL Taq DNA polymerase (Inclone biotech, Korea), 2.5 μL 10X reaction Buffer and 18.75 μL water. The quality of extracted genomic DNA was measured using a DNA engine thermal cycler (Biorad, Hercules, CA, USA). Cycling conditions were 5 min at 96 ℃ for denaturation followed by 36 cycles of 30 sec at 94 ℃, 30 sec at 55 ℃, 1 min at 72 ℃ for polymerization and 10 min at 72 ℃ for final extension. After PCR reaction for dCAPS markers, restriction enzyme reaction was performed in a volume of 10 μL containing 5 μL PCR product, 1 μL 10X restriction enzyme buffer and 0.3 μL (10 units) restriction enzyme. The mixtures were incubated

overnight at the optimal temperature. All PCR products were separated on 9 % polyacrylamide gels and stained with Ethidium bromide (EtBr) and visualized.

Sample preparation for metabolite analysis

Each collected adventitious root of PG, PQ and Hybrid were dried at 65 ℃ in the drying oven (HB-502L, Hanbaek Scientific Co, Korea). 1.0 mL 70 % methanol was applied to 25mg of powdered samples and ginsenosides were extracted twice for 90 min (total 180 min) using sonication at room temperature. Extracts were centrifuged at 1800 g for 5 min and the collected supernatant diluted with water (1→3) and filtered 0.2 μm RC-membrane filter (Minisart RC15; Sartorius Stedim Biotech, Germany). Samples were analyzed from three replicates in negative ion mode for repeatability and stability of measurement.

UPLC-QTOF/MS conditions for ginsenosides analysis

UPLC analysis utilized Waters ACQUITY UPLC ®system (Waters Co, Milford, MA, USA) with Waters ACQUITY UPLC BEH C18 (2.1 mm x 100mm, 1.7 μm) column. The column oven and sample tray were kept at 40 and 4 ℃, respectively. The mobile phase was composed of solvent A (water: formic acid = 100: 1, v/v) and solvent B (acetonitrile: formic acid = 100: 1, v/v). Both solvents were eluted at a flow rate of 0.3 mL/min and a gradient program was as follows: 0~9 min, B 20 %; 9~14 min, B 20~30 %; 14~17 min, B 30 %; 17~21 min, 30-32 %; 21~26 min, B 32~42.5 %; 26-29 min, B 42.5~90 %; 29~31min, B 90 %; 31~32 min, B 90~20 %; 32~35 min, B 20 %. The sample injection volume was 2.0 μL for each run.

MS analysis was performed using a Waters Xevo G2 QTOF MS collecting in negative ion mode and operated with electrospray ionization (ESI) interfere (ESI). The operating parameters of MS were set as follows: cone voltage, 45 V; capillary, 3.5 kV; source and desolvation temperatures, 120 and 350 ℃, respectively; flow of cone gas and desolvation gas, 50 and 800 L/hour respectively. Data were collected between 100 and 1500 m/z.

Statistical analysis

Experiments were conducted three times and results were analyzed as one data set. Statistically significant differences were followed by one-way analysis of variance (ANOVA); a, b and c mean statistical differences between data groups.

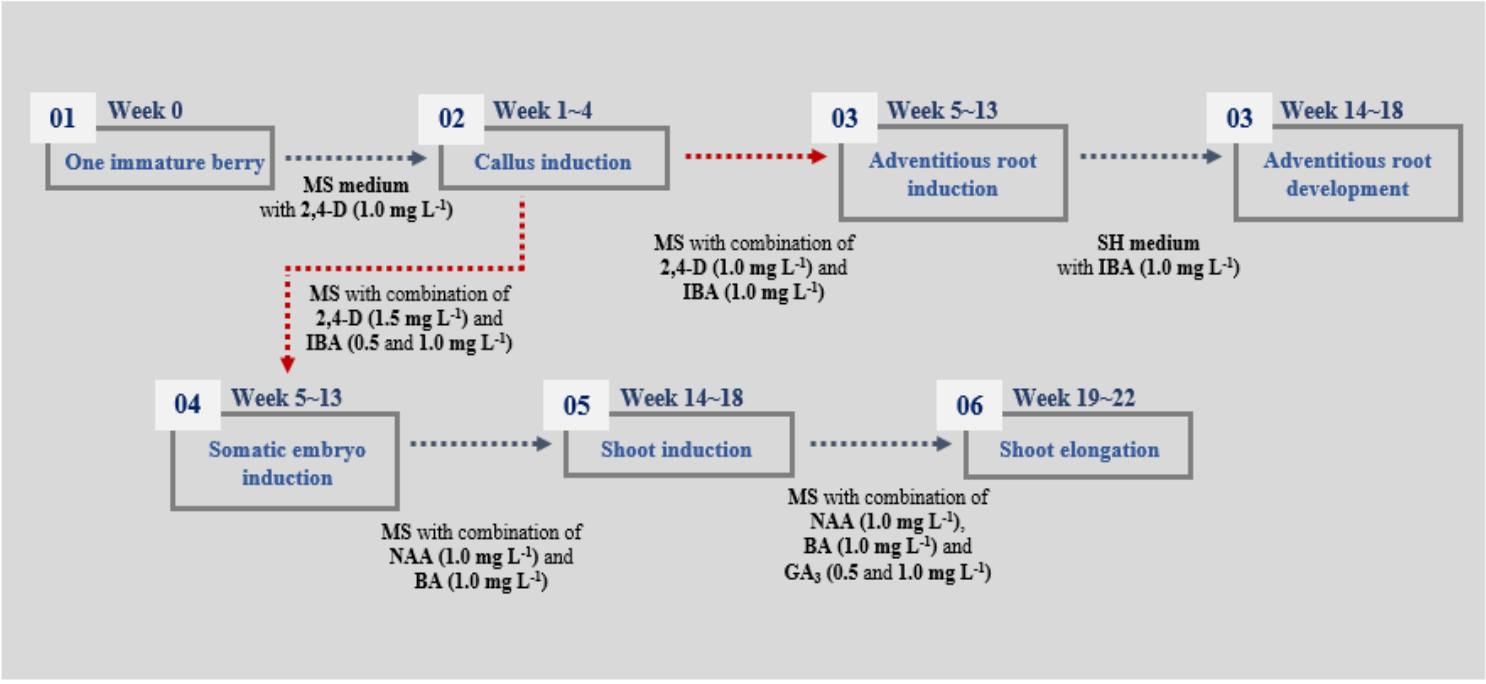


Figure 1. A flow chart of the protocol for tissue cultures of Hybrid. Step 1: A sterile flesh from one immature berry cultured on MS medium containing 1.0 mg L^{-1} 2,4-D to induce calli, Step 2: Proliferated calli were transferred onto MS medium containing 1.0 mg L^{-1} 2,4-D in combination with 1.0 mg L^{-1} IBA to induce adventitious roots and MS medium containing 1.5 mg L^{-1} 2,4-D in combination with 0.5 and 1.0 mg L^{-1} IBA to form somatic embryos. Step 3: Calli with adventitious roots were moved to liquid SH medium containing 2.0 mg L^{-1} IBA to develop well and get more biomass, Step 4: Calli with somatic embryos were transferred to MS medium containing 1.0 mg L^{-1} NAA in combination with 1.0 mg L^{-1} BA to develop somatic embryos and

induce shoots, Step 5: Developed somatic embryos with shoots were transferred to MS medium containing 1.0 mg L^{-1} NAA, 1.0 mg L^{-1} BA and 0.5 and 1.0 mg L^{-1} GA_3 and Stage 6: The shoots were elongated.

Table 1. Primers used in this study.

Ⅰ. Primers for amplifying *NPT Ⅱ* gene		
Gene	Forward primer (5'→3')	Reverse primer (5'→3')
NPT Ⅱ	ATCGGGAGCGGCGATACCGTA	GAGGCTATTCGGCTACTG
Ⅱ. SSR markers in analysis of *Panax ginseng* and *Panax quinquefolius*		
Marker	Forward primer (5'→3')	Reverse primer (5'→3')
gws454	AAAGGAATACAAGAAAGAGGGAGAA	TAAAGAATTTGGATCCACCTACAAA
gws1070	TTCCAAACATAAAAGAAAACTGACC	GTCTTGAAAACTTACCGAATTGAAA
PES0010	GTCTCGCAAAGAATGTCAGC	CTGCTTTTGCACCTCATAGC
PES0034	GCTTATCTTGCCTGATAATGTCCTA	CAACATGTAATTACGTCTCTCATGC
Ⅲ. dCAPS markers in analysis of *Panax ginseng* and *Panax quinquefolius*		
Gene	Forward primer (5'→3')	Reverse primer (5'→3')
rpl20	GTTTAAATTATTCCGGTGGATTCTT	GGTATCCTATAGTTATAGTAGATTAATCGA
ndhK	GTCCGCTTGTCTAGGACTCG	CAAAATTCAGTTATTTCAACTACATCAAT
rps15	ATCCAACCGACCAATTAATTCTTTA	TTGAAAGAGGAAAACAAAGACACCC
rpoC1	TATGACCGTCCCTCATCGGTTGTCG	CATTCAGATAGATGGGGGTAAACTA
ndhA	CTCGTAAACCACCTAAAAAGGAAT	TCGTTTATTCAGTATCGGACCATCG
ndhK	TTCCGGCTGTTAAAATTAGGTCAGC	TCTTTCAAATTGGTCAAGACTCTCT

RESULTS

1. Callus induction, proliferation, root production and somatic embryogenesis

For callus induction, only 2,4-D (auxin) was treated among plant growth regulators (PGRs). Embryos of PG / PQ and a sterile flesh from one immature berry of Hybrid cultured onto MS medium applied only 2,4-D (1.0mg L^{-1}). After 4 weeks, parts of explants were expanded and soft and friable calli were formed. We separated only calli from the explant and moved to fresh medium. Newly formed calli were soft and they were easliy broken down, but after a while they became hard. The first produced soft calli were transparent cream colored, and they turned into ivory color over time. In case of PQ and hybrid, the calli were turned purple throughout the surface when they were aged. During first 1 year, we continued to propagate the calli by periodic medium change (Figure 2).

After 8 weeks and 4 weeks of subculture once, calli were constantly grown in size on the medium containing only 1.0 mg L^{-1} 2,4-D as PGR (Figure 2). When proliferated calli were moved to MS medium with 1.0 mg L^{-1} 2,4-D and additional 0.5 and 1.0mg L^{-1} IBA, they formed green colored explants. Interestingly, the calli induced adventitious roots on MS medium with containing 1.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} IBA and the calli induced yellow-greenish post globular to heart shaped somatic embryos on MS medium of 1.5 mg L^{-1} 2,4-D in combination with 0.5 and 1.0 mg L^{-1} IBA. Adventitious roots could grow on the solid medium but they were transferred to liquid medium for greater root growth (Figure 3~5). For somatic

embryos, several embryos were formed per callus, and they were carefully moved to new media supplemented with NAA and BA (Figure 6).

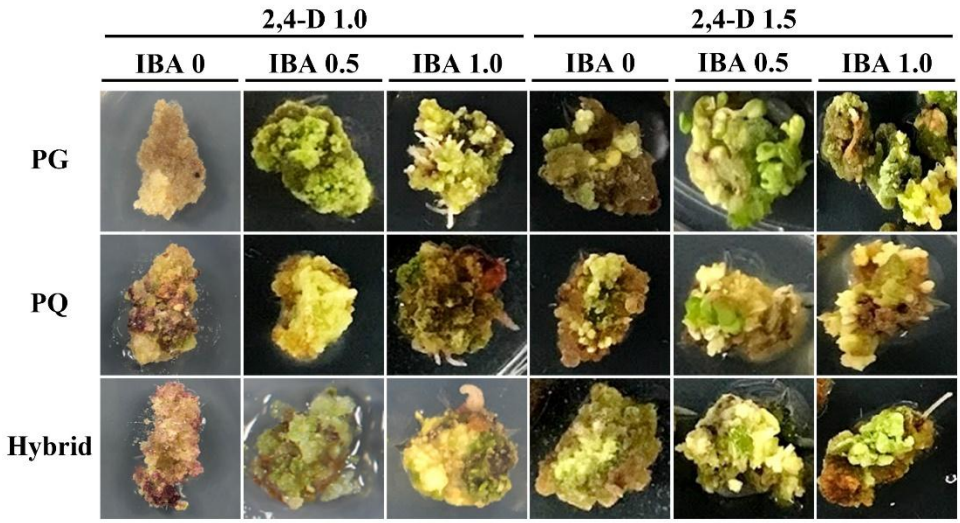


Figure 2. Morphological response from callus of PG, PQ and Hybrid on MS medium with combination of 2,4-D and IBA. The calli derived from embryos of PG and PQ as well as a flesh from one immature berry of Hybrid were induced adventitious roots and somatic embryos on MS medium containing different combinations of 2,4-D (1.0 and 1.5 mg L^{-1}) and IBA (0, 0.5 and 1.0 mg L^{-1}) after 8 weeks and 4 weeks of subculture once. Assays were performed at least three Petri dishes containing 12 calli per one.

2. Adventitious root development and establishment

Callus with adventitious roots were transferred to liquid SH medium with IBA. After 4 weeks, the fresh weight of PG, PQ and Hybrid calli increased as IBA concentration increased (Figure 3). There are great gaps in the fresh weight changes between without IBA and with IBA (2.0, 4.0 and 6.0 mg L^{-1}). Especially for Hybrid, it showed higher growth weight than its parents PG and PQ. Beside, PG and PQ showed normal adventitious root morphology, but Hybrid had a big difference in root morphology according to the growth media. On the medium without IBA, adventitious roots grew little and turned deep browned. However, as the concentration of IBA increased, adventitious roots gave rise to callus formation, shaping into a ball and shortening the length of new root (we selected the longest one from each group, respectively) even the solution in the medium became turbid (Figure 4 and 5). Consequently, liquid SH medium with 2.0 mg L^{-1} IBA was selected as the best medium to proliferate the adventitious root of Hybrid.

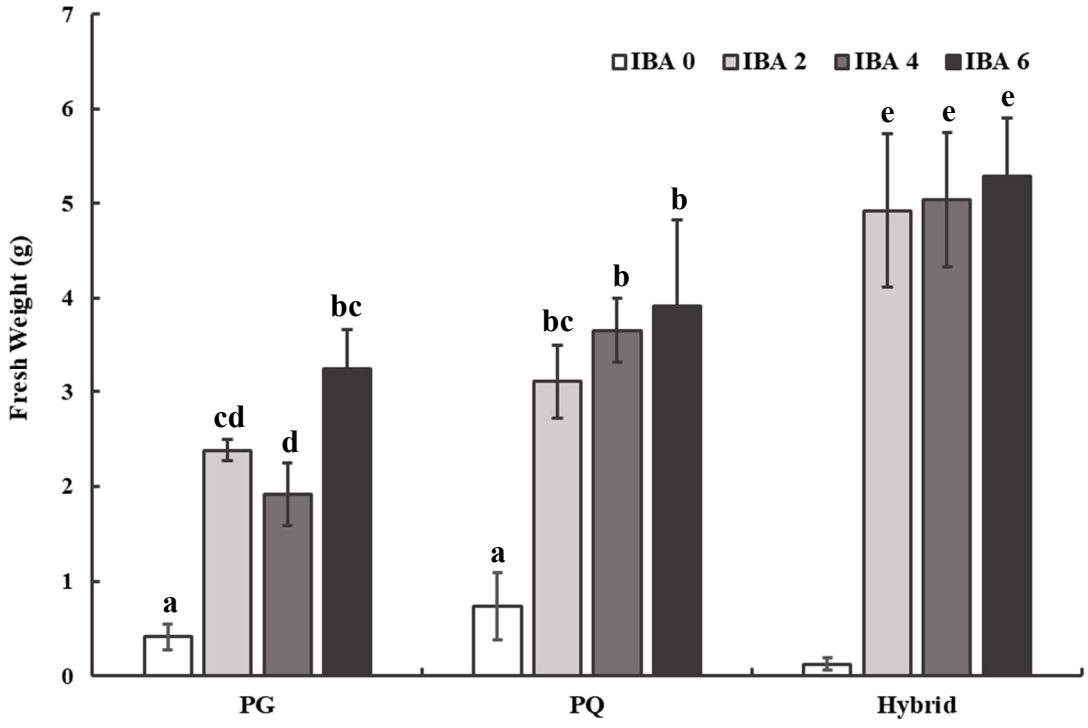


Figure 3. Comparison of growth weight on PG, PQ and Hybrid adventitious roots in liquid SH medium with different concentrations of IBA (0, 2.0, 4.0 and 6.0 mg L^{-1}) after 4 weeks. Data were obtained as mean ± SD (n=3). Means and values with significant difference were identified by the Duncan's post hoc test.

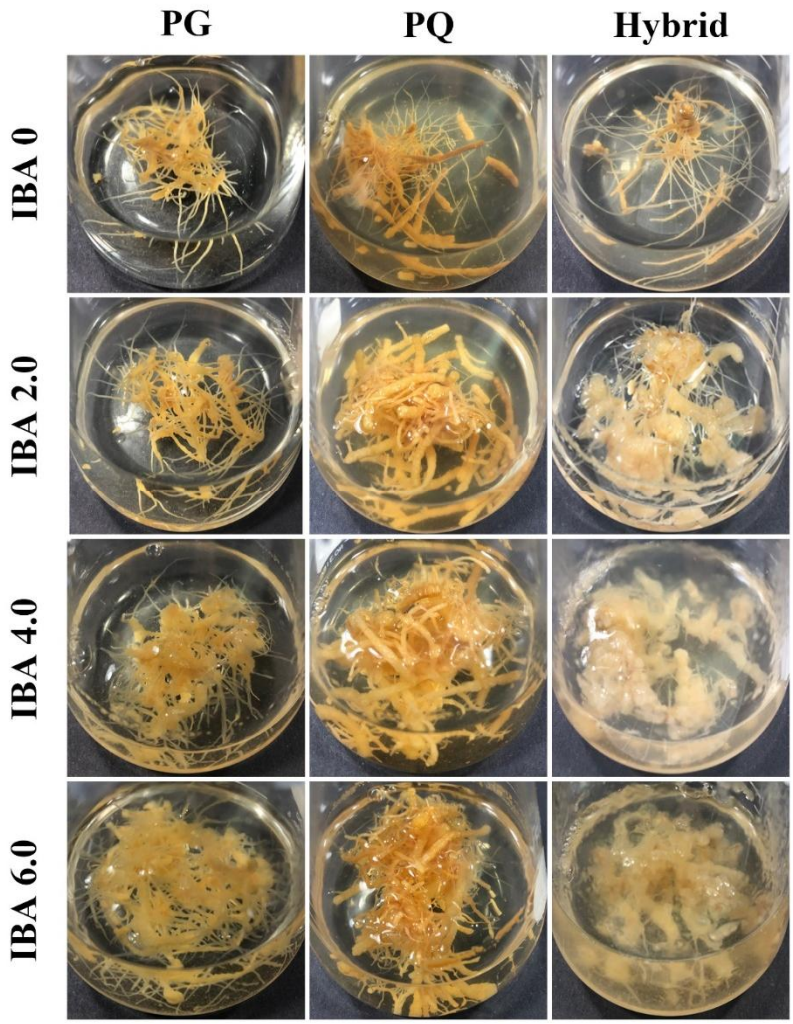


Figure 4. Morphological response of PG, PQ and Hybrid adventitious roots in liquid SH medium with different concentrations of IBA (0, 2.0, 4.0 and 6.0 mg L^{-1}) after 4 weeks.

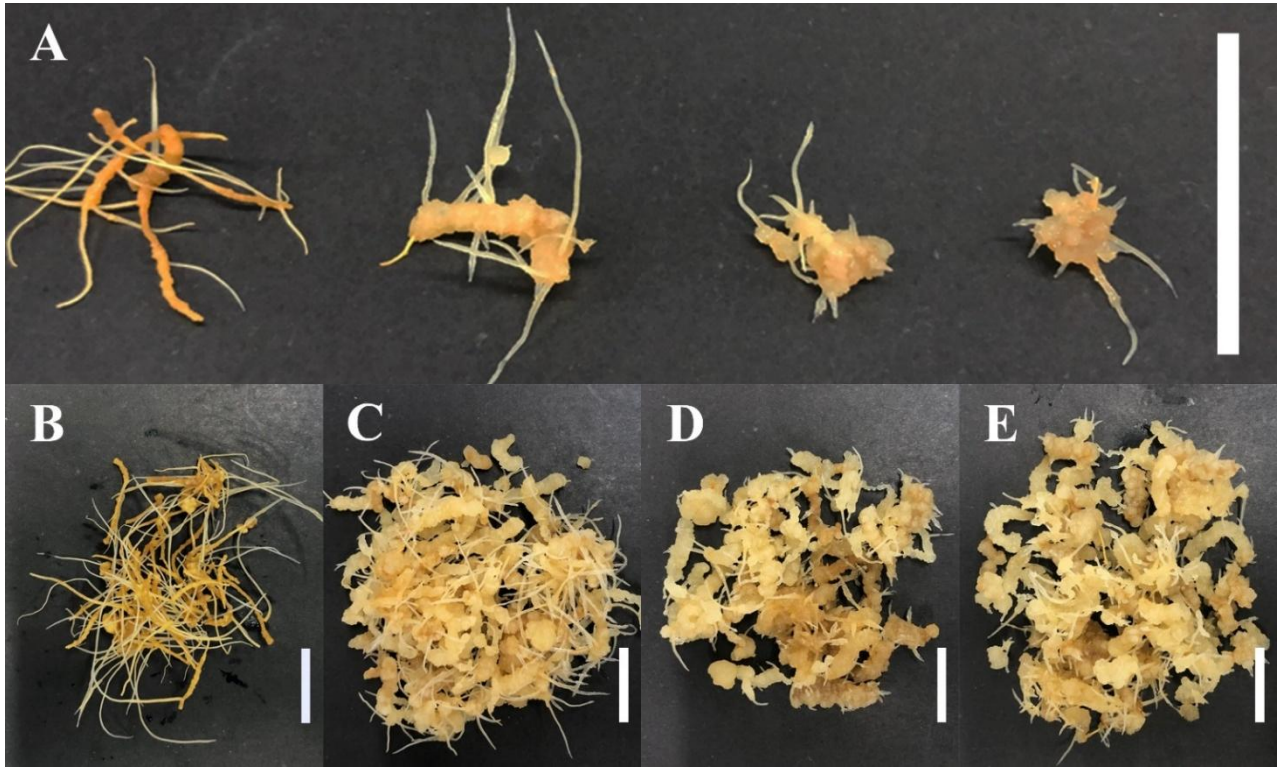


Figure 5. Morphology of Hybrid adventitious root in liquid SH medium with different concentrations of IBA (0, 2.0, 4.0 and 6.0 mg L^{-1}) after 4 weeks. (A) Comparison of individual adventitious root from different IBA treatments. SH medium supplemented (B) without IBA, (C) with 2.0 mg L^{-1} IBA, (D) with 4.0 IBA and (E) with 6.0 mg L^{-1} IBA. (*bars* = 2 cm)

3. Shooting of somatic embryos and plantlet conversion

We transferred callus with somatic embryos to new medium supplemented only 1.0 mg L^{-1} NAA or combination with 0.5 and 1.0 mg L^{-1} BA (Figure 6A). On MS media containing only NAA, the calli showed no germination response and surrounding edges of calli were brown in color. When NAA and BA were used together (0.5 and 1.0 mg L^{-1}, respectively), well-developed embryos were swelled in green and induced shoots. Explant on culture containing 1.0 mg L^{-1} BA produced a greater number of shoots than lower concentration of 1.0 mg L^{-1} BA. Upon transfer to medium with NAA, BA and GA_3, all shoots were elongated. In the other words, calli tend to produce shorter and thicker shoots without GA_3, and they produced slender and more elongated shoots with GA_3 (Figure 6B). It is necessary to separate each shoot from a callus of multiple shoots. When shooting somatic embryos were not separated within a callus of multi-individuals, shoots did not grow well. So we had to detach each one or two individuals from multi-somatic embryos using a razor blade. Overall, obtaining an optimal concentration of auxin (NAA), cytokinin (BA) and GA_3 was important to develop shoots from somatic embryos.

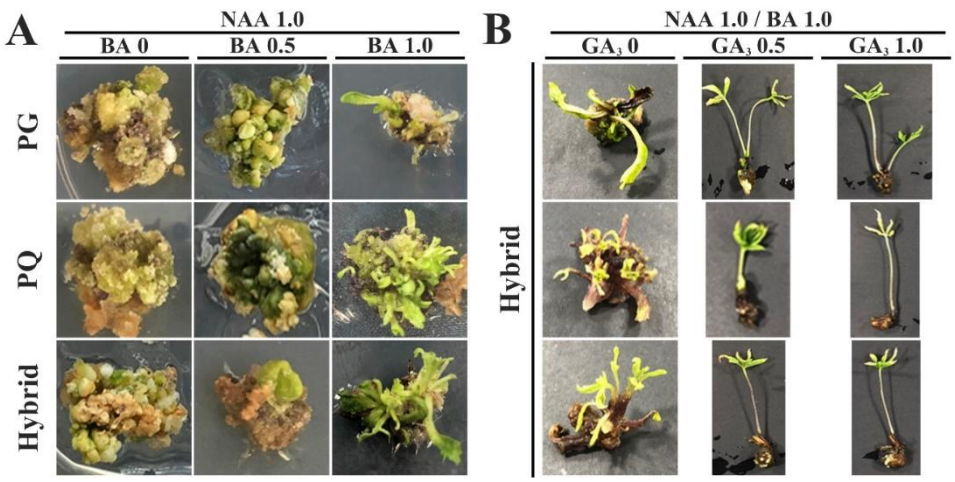


Figure 6. Morphological response of somatic embryos in PG, PQ and Hybrid on MS medium with combination of NAA, BA and GA_3. (A) Somatic embryos were induced multiple shoots on MS medium containing different combinations of NAA (1.0 mg L^{-1}) and BA (0, 0.5 and 1.0 mg L^{-1}) and (B) multiple shoots were elongated to plantlets on MS medium containing different combinations of NAA (1.0 mg L^{-1}), BA (1.0 mg L^{-1}) and GA_3 (0.5 and 1.0 mg L^{-1}) after 8 weeks and 4 weeks of subculture once. Assays were performed at least three Petri dishes containing 12 calli per one.

4. *Agrobacterium.rhizogenes*-mediated transformation and hairy root induction from callus of Hybrid

After co-cultured with *A.rhizogenes* for 8 weeks on a half MS medium containing kanamycin (50 mg L^{-1}) and cefotaxime (250 mg L^{-1}), hairy roots were induced from the wound-infected and survived calli (Figure 7A). The number of hairy roots per explant (callus) was 2.96 and hairy root induction rate was 44.58% (not shown). When hairy roots grew more than 5 cm in length, we cut only hairy root from the callus and transferred to liquid SH medium with kanamycin (50 mg L^{-1}) and cefotaxime (250 mg L^{-1}), and incubated for 4 weeks. The transformation of the genes in binary vector into hairy roots were confirmed through primers that can amplify the *NPT Ⅱ* gene (Figure 7B). After 4 weeks of incubation in liquid SH medium with antibiotics, hairy roots were moved to either IBA-free or IBA-containing (3.0 mg L^{-1}) liquid SH medium. For comparison, adventitious roots were also maintained either IBA-free or IBA-contained (3.0 mg L^{-1}) liquid SH medium. After another 4 weeks of culture, the difference was found between hairy root and adventitious root both grown in IBA-contained SH medium. At the same concentration of IBA (3.0 mg L^{-1}), it was observed that more newly formed roots were observed in hairy root than adventitious root. Although there was no noticeable growth in adventitious root in the IBA-free liquid SH medium (Figure 3), hairy roots grew well even in IBA-free SH media, and yielded similar fresh weight between IBA-free or IBA-contained liquid SH. Fresh weight values were as follows: Hairy root with IBA; 12.18 g, Hairy root without IBA; 10.82 g, Adventitious root with IBA; 4.9 g, Adventitious root without IBA; 0.12 g. Overall,

hairy root culture system showed higher growth rate than adventitious root, and does not require auxin for their growth.

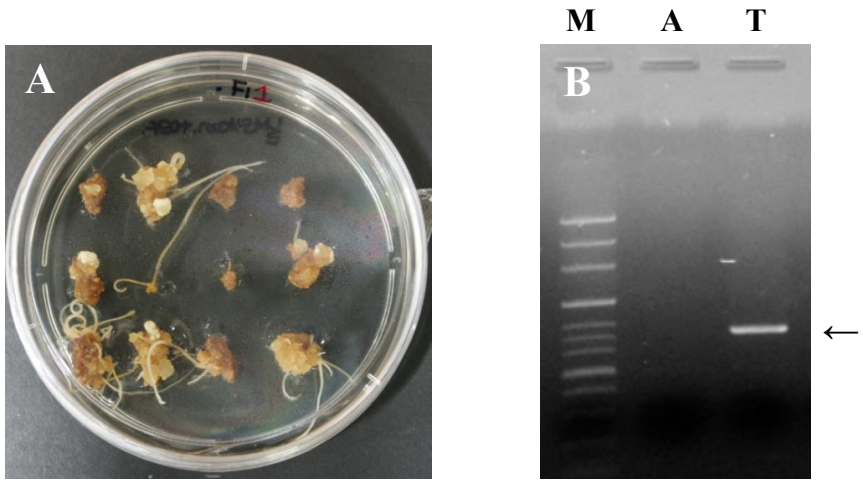


Figure 7. *A.rhizogenes* R1000 induced hairy root from callus of Hybrid. (A) Front view on a half MS containing kanamycin (50 mg L^{-1}) and cefotaxime (250 mg L^{-1}). (B) Detection of *NPT Ⅱ* gene in pBI121 from transgenic hairy roots of Hybrid by PCR (*M* molecular markers, *A* Adventitious root, *T* Transgenic hairy root and *arrowhead* expressed 700 bp fragment size of *NPT Ⅱ* gene).

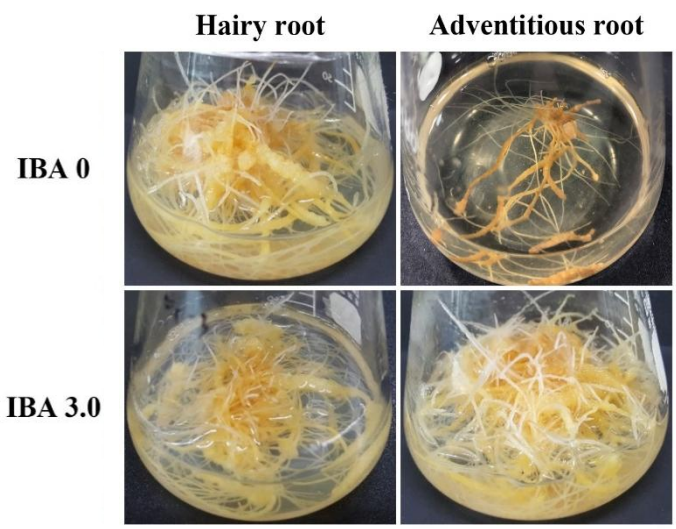


Figure 8. Morphology of hairy root and adventitious root from Hybrid in medium without or with 3.0 mg L^{-1} IBA.

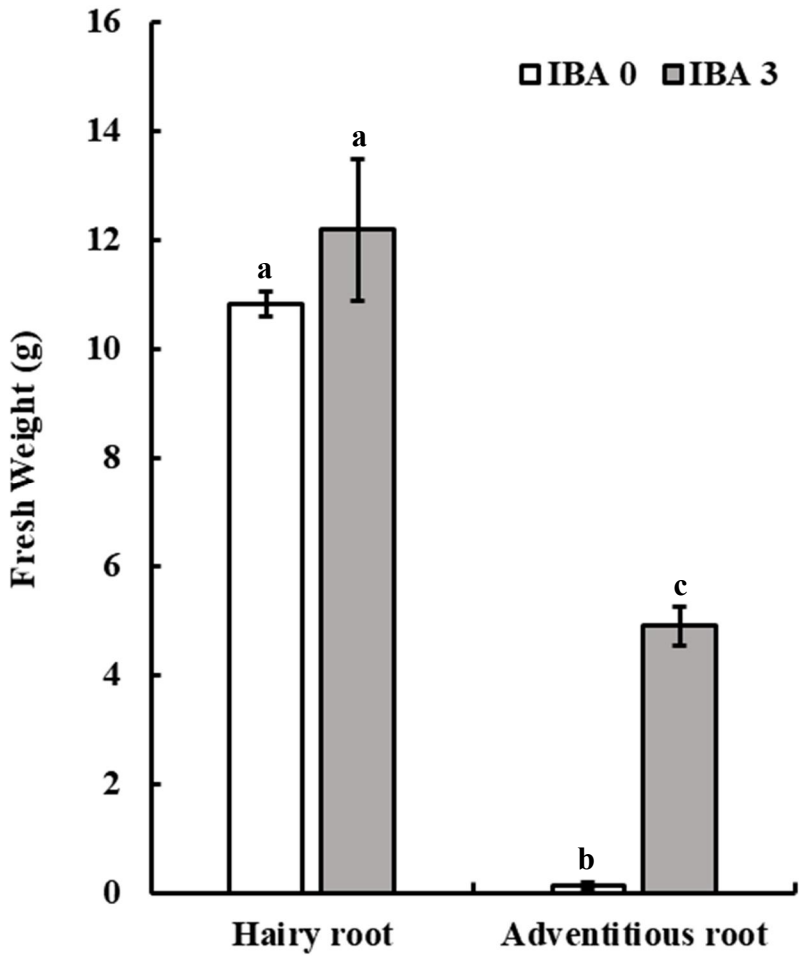


Figure 9. Growth weight of Hybrid hairy root and adventitious root in liquid SH medium without IBA or with 3.0 mg L^{-1} IBA after 4 weeks. Data were obtained as mean ± SD (n=3). Means and values with significant difference were identified by the Duncan's post hoc test.

5. SSR and dCAPS analysis for discriminative validation of Hybrid and Parents

We could validate parents of Hybrid using SSR markers and identify maternal line of Hybrid using dCAPS markers. Four SSR markers designed to detect variable numbers of specific repeat motif among ginseng species range in size from 100bp to 290 bp, and primers were designed to amply these SSR markers (Table 1). Electrophoresis of the PCR products showed different band patterns according to PG and PQ. Each primer set yielded specific target bands according to Parents (PG and PQ), and Hybrid shared all or some bands with its parents (Figure 10). In gws454 and gws1070, PG (lane 1 and 4) and PQ (lane 3 and 6) showed only one clear band. Instead, Hybrid (lane 2 and 5) had both bands from PG and PQ (Figure 10). In PES0010 and PES0034, PG (lane 7 and 10) and PQ (lane 9 and 12) showed multi bands, but some of these bands were not common between PG and PQ. Again, Hybrid shared bands from the parents. Overall, these results referred to typical interspecies hybrid polymorphism and clearly showed that Hybrid is an offspring of PG and PQ.

To identify the maternal line of Hybrid, 6 markers were selected from specific SNP targets. These dCAPS markers could distinguish the markers derived from PG or PQ after being cut by restriction enzyme. The comparison of 6 markers derived from *rpl20*, *ndhK*, *rps15*, *rpoC1*, *ndhA* and *ndhK* , which are coded by chloroplast genome, confirmed that maternal line of Hybrid is PG (Figure 11).

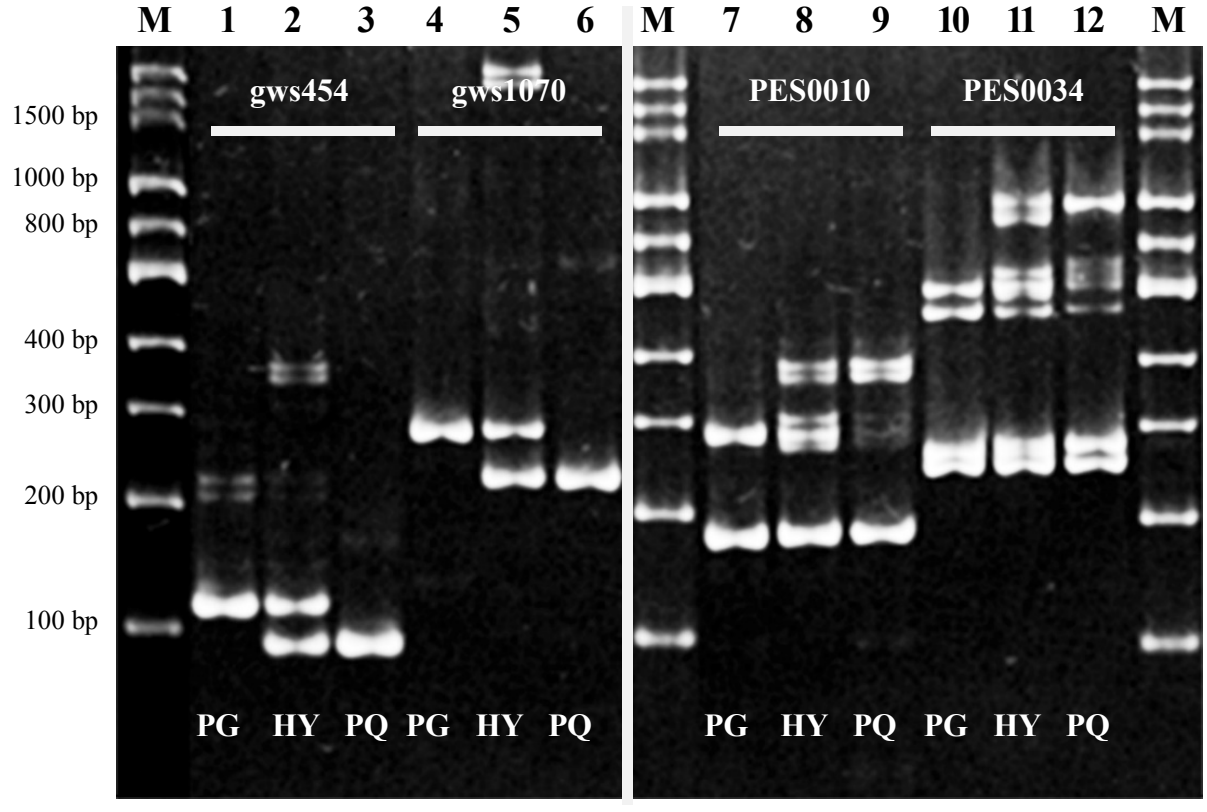


Figure 10. DNA profiles of PG, HY (Hybrid) and PQ using SSR markers derived from nuclear genome.

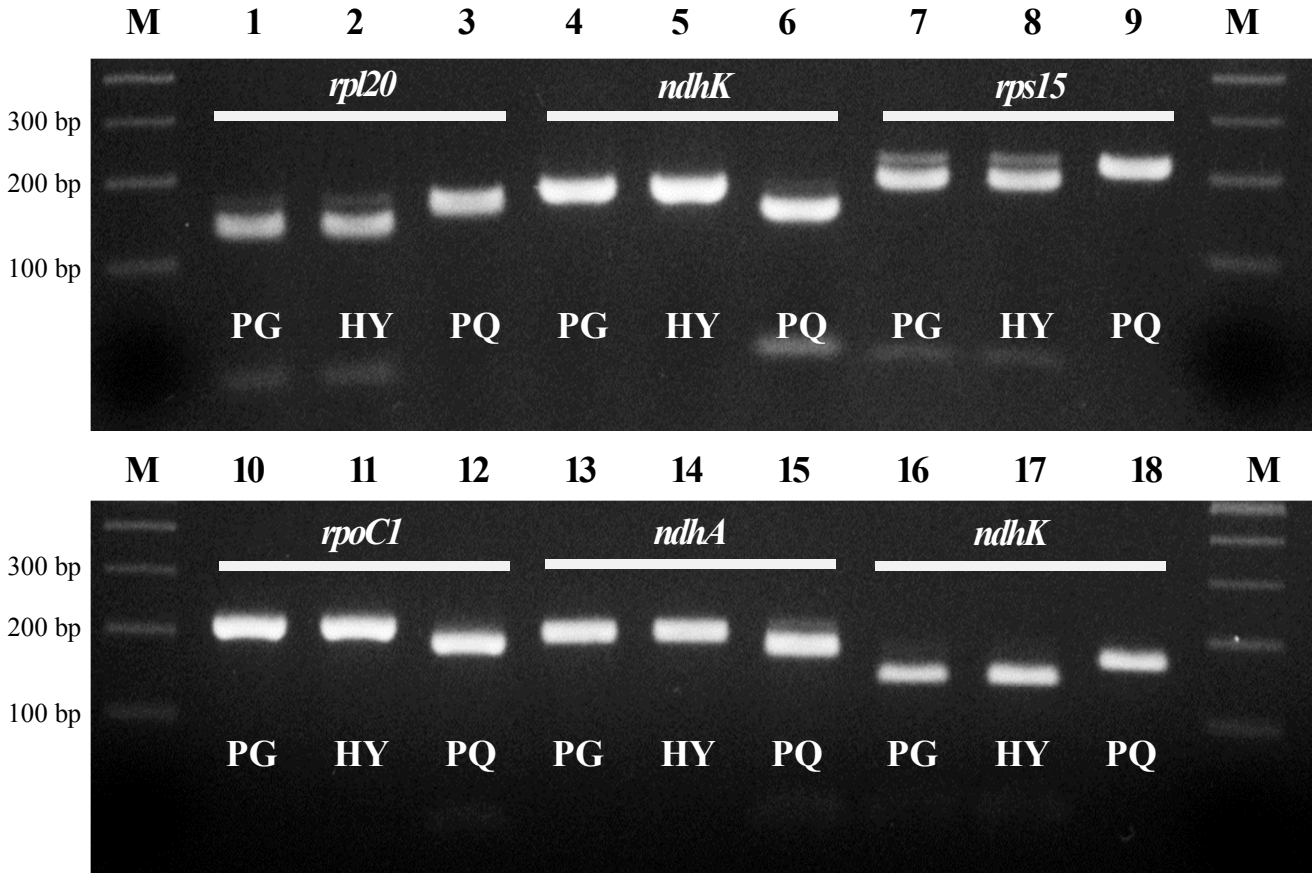


Figure 11. DNA profiles of PG, HY (Hybrid) and PQ using dCAPS markers derived from chloroplast genome.

6. Comparison of ginsenosides in adventitious roots of Hybrid, PG and PQ

We performed secondary metabolite profiling in adventitious roots of PG, PQ and Hybrid using UPLC-QTOF/MS. The ginsenosides were identified using in-house library constructed with authentic standards (Figure 12). The information of 53 ginsenoside peaks, the peak number, name of compound, retention time (RT) and major fragment ion (m/z), are addressed in Table 2. The total distribution of 46 ginsenosides could be confirmed with the Venn diagram excluding unknown ginsenosides (Figure 13).

The number of ginsenosides for individual samples were as follows: PG; 33, PQ; 29, Hybrid; 46. They had different numbers of ginsenosides inter species. In detail, PG and PQ commonly quantified as follows: G-Rg_1, G-Re, 6'-O-Acetyl-G-Rg_1 isomer, G-Rg_2, G-Rb_1, m-G-Rb_1, G-Rc, G-Ro, m-G-Rb_1 isomer, m-G-Rc, G-Ro isomer, G-Rb_2, G-Rb_3, m-G-Rb_2, m-G-Rb_3, Zingibroside R_1, G-Rd, m-G-Rd, Gypenoside XVII, m-G-Rd isomer, Chikusetsusaponin Iva and Chikusetsusaponin Iva isomer. The following 11 ginsenosides (red-colored) in PG were specifically observed: FloralG-M/FloralG-N, m-G-Re, G-Rf, G-F_3, 6'-O-Acetyl-G-Rg_1 isomer, m-G-Rb_1 isomer, m-G-Rb_2/m-G-Rb_3/m-G-Rc isomer, Acetyl-G-Rd, Compound Mc1, Gypenoside IX and G-Rg_3. and 7 ginsenosides (green and blue-colored) in PQ was uniquely observed: notoginsenoside R_1 isomer, Pseudoginsenoside F_{11} isomer, 6'-O-Acetyl-G-Rg_1 isomer, Pseudoginsenoside F_{11}, Chikusetsusaponin IV, m-G-Rb_2/m-G-Rb_3/m-G-Rc isomer and Pseudoginsenoside RT_5. In the case of the Hybrid, almost all ginsenosides of PG and PQ, except only one, which is Pseudoginsenoside RT_5 (blue-colored) and also included 6 ginsenosides that are

notoG-R_1, Floralquinquenoside D, Notoginsenoside Rw_2, G-Rh_1, 6'-O-Acetyl-G-Rh_1, 6'-O-Acetyl-G-Rg_2 isomer (yellow-colored).

Consequentially, 45, 33 and 29 ginsenoside contents were detected in Hybrid, PG and PQ respectively using LC-ESI-MS/MS in multiple reaction monitoring (MRM) acquisition mode (Figure 13). Similar to aspect of SSR marker test, PG and PQ showed different ginsenoside profiles; especially earlier research showed pseudoginsenoside F_{11} was not detected in PG and ginsenoside Rf was a lot more abundant in PG than in PQ [13] and in our result, Hybrid had both pseudoginsenoside F_{11} and ginsenoside Rf (Figure 15). These results indicated Hybrid shared PG and PQ in secondary metabolites in addition produced new ginsenosides.

Table 2. Ginsenosides identified from standards and ginseng samples **(**In-house library).

No	Identity	Ret. Time	Major fragment ions (m/z)
1	notoG-R_1 isomer	5.1665	931.5261
2	notoG-R_1	5.6911	931.5264
3	Floral-G-M/Floral-G-N	5.7716	1077.5846
4	G-Rg_1	8.1538	845.4901
5	G-Re	8.5745	945.5420
6	Pseudoginsenoside F_{11} isomer	11.0325	799.4845
7	6'-O-Acetyl-G-Rg_1	11.6798	841.4954
8	m-G-Re	13.0524	1031.5428
9	6'-O-Acetyl-G-Rg_1 isomer	13.9105	841.4950
10	Floralquinquenoside D	14.5063	815.4787
11	NotoG-Rw_2	15.6804	785.4687
12	Pseudoginsenoside RT_5	15.9531	653.4265
13	G-Rf (PG)	16.0429	799.4839
14	Pseudoginsenoside F_{11} (PQ)	16.0429	799.4839
15	G-F_3	16.8721	769.4735

16	Yesanchinoside D (=notoginsenoside Rt)	17.0801	841.4939
17	G-Rh_1	18.1662	637.4318
18	G-Rg_2	18.2290	783.4890
19	unknown 2	18.8468	857.4900
20	G-Rb_1	18.8691	1107.5950
21	m-G-Rb_1	19.7838	1193.5959
22	6'-O-Acetyl-G-Rh_1	19.8761	679.4410
23	6'-O-Acetyl-G-Rg_2 isomer	20.0025	825.5000
24	unknown 3	20.1757	1087.5328
25	G-Rc	20.2307	1077.5837
26	G-Ro	20.5261	955.4900
27	m-G-Rb_1 isomer	20.7011	1193.5966
28	unknown 4	21.1778	1087.5332
29	m-G-Rc	21.1784	1163.5844
30	G-Ro isomer	21.2894	955.4908
31	unknown 6	21.5421	1279.5965
32	m-G-Rb_1 isomer	21.7896	1193.5966
33	G-Rb_2	21.8486	1077.5845
34	G-Rb_3	22.3579	1077.5837
35	Chikusetsusaponin IV	22.5632	925.4791
36	m-G-Rb_2	22.6808	1163.5857
37	m-G-Rb_3	23.0072	1163.5840

38	zingibroside R_1	23.6113	793.4374
39	m-G-Rb_2/m-G-Rb_3/m-G-Rc isomer-1	23.7178	1163.5841
40	G-Rd	24.0362	945.5417
41	m-G-Rb_2/m-G-Rb_3/m-G-Rc isomer-2	24.1556	1119.5948
42	m-G-Rd	24.4545	1031.5427
43	Gypenoside XVII	25.244	945.5421
44	Acetyl-G-Rd	25.3303	987.5525
45	m-G-Rd isomer	25.9288	1031.5427
46	Compound Mc1 = notoginsenoside Fe	26.0063	915.5314
47	Gypenoside IX	26.5751	915.5319
48	unknown 7	26.7616	915.5324
49	G-Rg_3	27.6415	783.4886
50	Chikusetsusaponin Iva	27.8155	793.4369
51	unknown 10	27.8341	925.4802
52	Chikusetsusaponin Iva isomer	28.0458	793.4373
53	unknown 12	28.3280	763.4266

G: ginsenoside

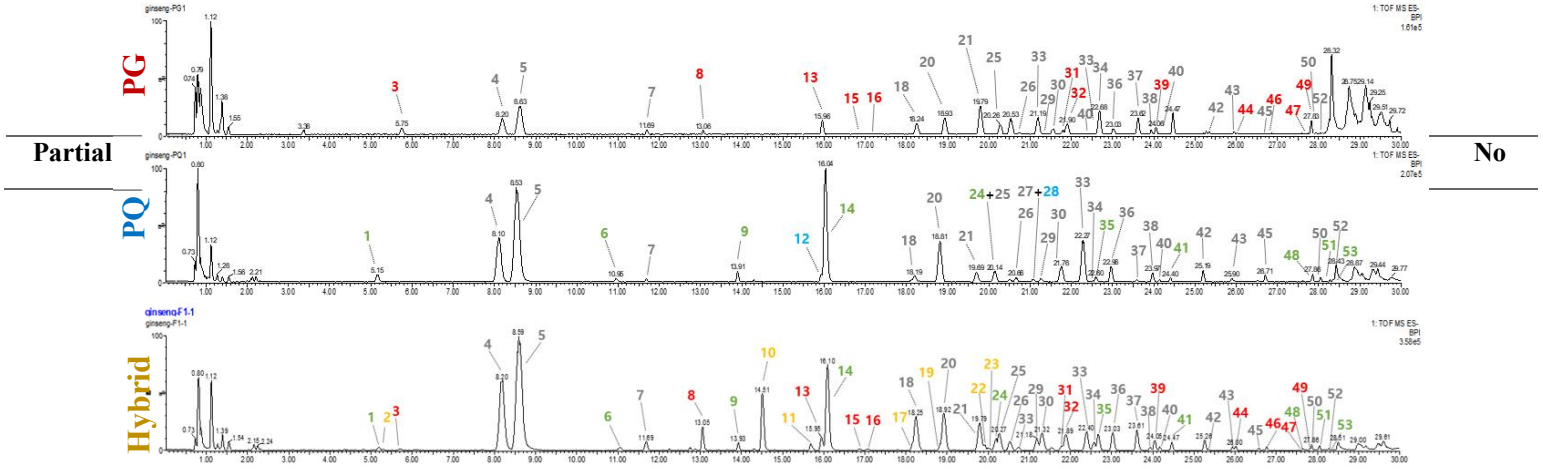


Figure 12. The base peak intensity (BPI) chromatogram of ginsenosides in adventitious root of (A) PG, (B) PQ and (C) Hybrid.

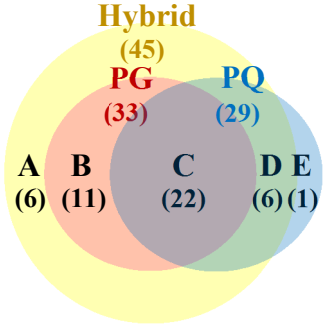
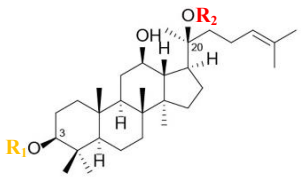


Figure 13. Comparative ginsenosides among PG, PQ and Hybrid using the Venn diagram.

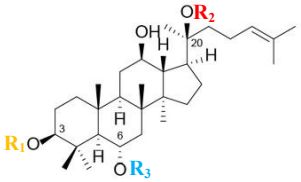
area		
A	notoG-R_1, Floralquinquenoside D, Notoginsenoside Rw_2, G-Rh_1, 6'-O-Acetyl-G-Rh_1, 6'-O-Acetyl-G-Rg_2 isomer	6
B	FloralG-M/FloralG-N, m-G-Re, G-Rf (PG), G-F_3, 6'-O-Acetyl-G-Rg_1 isomer, m-G-Rb_1 isomer, m-G-Rb_2/m-G-Rb_3/m-G-Rc isomer, Acetyl-G-Rd, Compound Mc1, Gypenoside IX, G-Rg_3	11
C	G-Rg_1, G-Re, 6'-O-Acetyl-G-Rg_1 isomer, G-Rg_2, G-Rb_1, m-G-Rb_1, G-Rc, G-Ro, m-G-Rb_1 isomer, m-G-Rc, G-Ro isomer, G-Rb_2, G-Rb_3, m-G-Rb_2, m-G-Rb_3, Zingibroside R_1, G-Rd, m-G-Rd, Gypenoside XVII, m-G-Rd isomer, Chikusetsusaponin Iva, Chikusetsusaponin Iva isomer	22
D	notoginsenoside R_1 isomer, Pseudoginsenoside F_{11} isomer, 6'-O-Acetyl-G-Rg_1 isomer, Pseudoginsenoside F_{11} (PQ), Chikusetsusaponin IV, m-G-Rb_2/m-G-Rb_3/m-G-Rc isomer	6
E	Pseudoginsenoside RT_5	1
Total		46

Table 3. Identity and number of sharing or unique ginsenosides among PG, PQ and Hybrid.

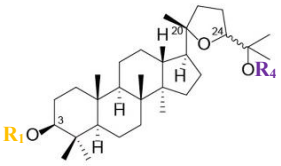
A



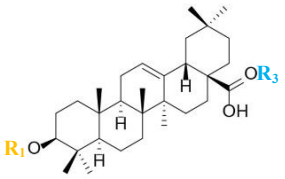
Protopanaxadiol (**PPD**)



Protopanaxatriol (**PPT**)



Ocotillol (24*R*-) or Ocotillol Ⅱ (24*S*-) (**OT**)



Oleanolic acid (**OA**)

B

Figure 14. Structures of 13 major ginsenosides in adventitious roots of PG,

No	Identity	Molecular formula	Aglycone	R_1	R_2	R_3	R_4
4	G-Rg_1	$C_{42}H_{72}O_{14}$	PPT	H	Glc	Glc	-
5	G-Re	$C_{48}H_{82}O_{18}$	PPT	H	Glc	Glc^{2}-^{1}Rha	-
13	G-Rf (PG)	$C_{42}H_{72}O_{14}$	PPT	H	H	Glc^{2}-^{1}Glc	-
14	Pseudoginsenoside F_{11} (PQ)	$C_{42}H_{72}O_{14}$	OT	-	-	-	Glc^{2}-^{1}Rha
17	G-Rh_1	$C_{36}H_{62}O_{9}$	PPT	H	H	Glc	-
18	G-Rg_2	$C_{42}H_{72}O_{13}$	PPT	H	H	Glc^{2}-^{1}Rha	-
20	G-Rb_1	$C_{54}H_{92}O_{23}$	PPD	Glc^{2}-^{1}Glc	Glc^{6}-^{1}Glc	-	-
25	G-Rc	$C_{53}H_{90}O_{22}$	PPD	Glc^{2}-^{1}Glc	Glc^{6}-^{1}Ara(f)	-	-
26	G-Ro	$C_{48}H_{76}O_{19}$	OA	$GlcA^{2}$-^{1}Glc	-	(28)-Glc	-
33	G-Rb_2	$C_{53}H_{90}O_{22}$	PPD	Glc^{2}-^{1}Glc	Glc^{6}-^{1}Ara(p)	-	-
34	G-Rb_3	$C_{53}H_{90}O_{22}$	PPD	Glc^{2}-^{1}Glc	Glc^{6}-^{1}Xyl	-	-
40	G-Rd	$C_{48}H_{82}O_{18}$	PPD	Glc^{2}-^{1}Glc	Glc	-	-
49	G-Rg_3	$C_{42}H_{72}O_{13}$	PPD	Glc^{2}-^{1}Glc	H	-	-

PQ and Hybrid (A) Classification according to attached glycosides (B) G: ginsenoside, Glc: glucose, Rha: rhamnose, Ara(f): arabinofuranose, Ara(p): arabinopyranose, GlcA: glucuromic acid.

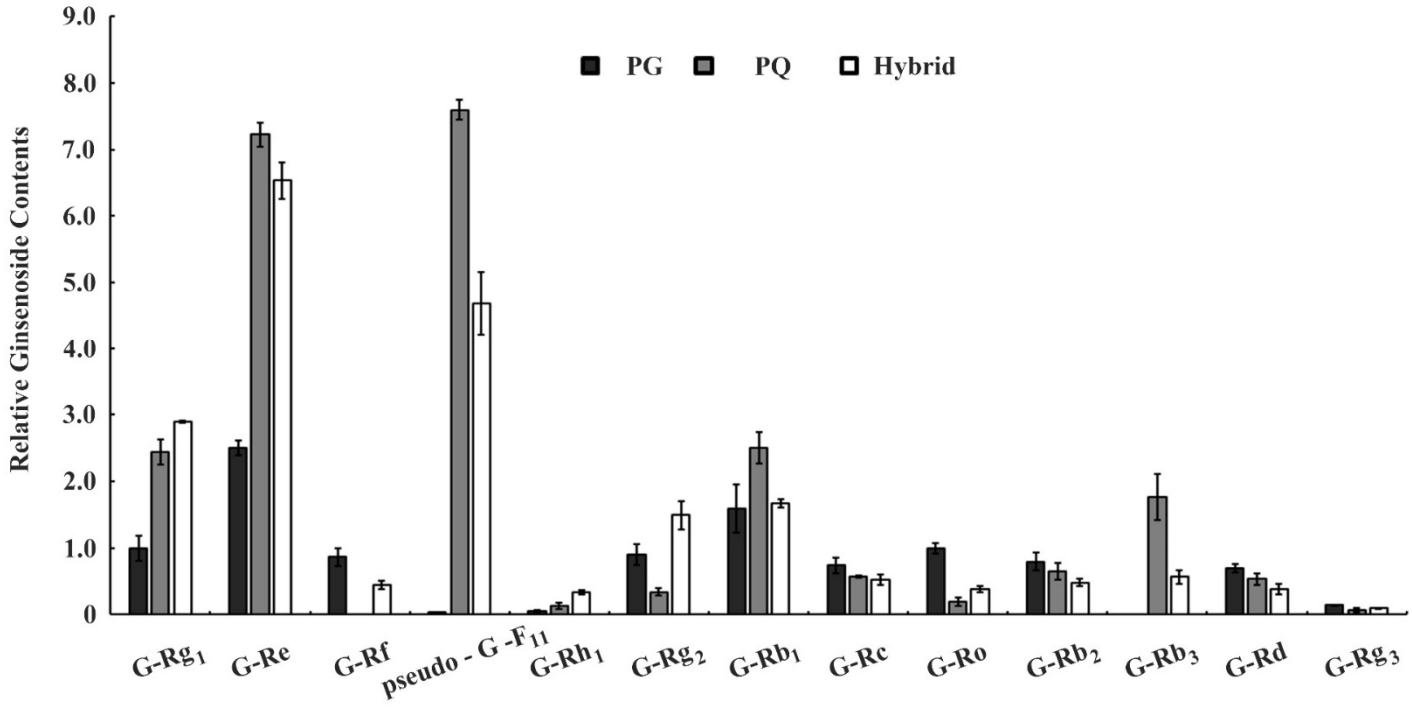


Figure 15. Relative ginsenoside contents of 13 major ginsenosides in adventitious roots of PG, PQ and Hybrid.

DISCUSSION

1. Various tissues development from callus

To establish the tissue culture system of Hybrid which is an interspecies hybrid of *Panax ginseng* and *Panax quinquefolius*, callus derived from one immature berry was induced to different tissues at various types and concentrations of plant growth regulators. As shown in Figure 2, we examined the changes of calli on medium supplemented with 2,4-D and IBA. On medium containing 1.0 mg L^{-1} of both 2,4-D and IBA, adventitious roots were induced and grown. Then, only adventitious roots were transferred into SH medium supplemented with IBA to proliferate.

The effect of exogenously applied IBA proved that this was the most efficient treatment because it interrupted callus formation, earlier root formation and also induced higher number of roots in *Prunus* spp. [27]. The higher concentration of IBA, the more adventitious roots of PG and PQ were generated. On the other hand, adventitious root culture of Hybrid line for yield improvement showed that as the IBA concentration increased, adventitious roots gave rise to callus formation, shortening the length of the induced root (Figure 4 and 5). Therefore, these results imply that Hybrid may require proper concentration of IBA.

The reason why the induction basal medium (MS medium) and the proliferating medium (SH medium) differ from each other were as follows: [28] [29] reported that MS basal medium containing NH_4NO_3 affected adventitious rooting process in the presence of auxin. Since mitogen-activated protein in kinase

(MAPK) transduction cascades were important mediates in signal transmission and were activated induction of adventitious root by auxin. [30] reported that SH medium which omitted NH_4NO_3 in MS medium formed more adventitious root than MS and a half MS medium. Moreover, ginsenosides were also enhanced by the omission of NH_4NO_3 from MS medium.

Previous researches mentioned that 2,4-D induced callus and somatic embryos in an appropriate concentration [15] [31] [32] [33] [34]. Although MS medium containing 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BA have been reported [35] [36] for somatic embryo germination, Hybrid induced adventitious roots in these conditions (not shown). Rather, the condition of MS medium containing 1.0 mg L^{-1} NAA in combination with 0.5 and 1.0 mg L^{-1} BA germinated the somatic embryos. We found that transgenic hairy roots enable more production of fresh weight than adventitious roots through *Agrobacterum rhizogenes*-mediated transformation and hairy root could grow well without IBA. Overall, only one immature berry of Hybrid was developed to various tissues such as callus, adventitious root, somatic embryo, plantlet and hairy root.

2. SSR and dCAPS markers for authentication of Hybrid and Parents.

DNA markers have been used as a key technique as a fast, easy, accurate and practical method to identify plant species. SSR markers were derived from EST, relatively conserved regions in the genome [25]. Compared to gws454 and gws1070, PES0010 and PES0034 showed distinct multi-bands or paralogous gene copies (Figure 10). This shows highly replicated polyploidy levels since both PG

and PQ are allotetraploids. Nevertheless, all four SSR markers lead to shared bands from the parents.

Chloroplast genome is highly conserved with multiple copies, having their own genome by maternal inheritance [37] and interspecies variation [38]. The dCAPS markers were able to correctly distinguish the maternal line of Hybrid and showed that PG and Hybrid had the same digestion patterns.

3. Ginsenoside profiling in adventitious root of Hybrid, PG and PQ

Hybrid, PG and PQ were compared based on ginsenoside profiling using UPLC-QTOF/MS analysis. UPLC could separate resolution compounds in a short period of time and QTOF/MS was useful to detect ginsenosides through the mass scan of whole ions [39]. Compared to previous studies dealing with eight ginsenosides, 46 ginsenosides were identified from in-house library and in addition, six new ginsenosides detected in adventitious root of Hybrid. It is possible that new pathways were created in Hybrid by combining some materials or enzymes derived from PG and PQ. Otherwise, it could be new six ginsenosides derived from different populations of *Panax ginseng* and *Panax quinquefolius*. Still, Hybrid contained most ginsenosides of PG and PQ [40].

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ABSTRACT IN KOREAN

고려인삼 (*Panax ginseng* C. A. Meyer, PG) 과 미국삼 (*Panax quinquefolius* L., PQ) 은 의약 및 기능성 식품의 원료로써 널리 사용되어 왔다. 이들은 이질사배체로써 계통발생관계가 가깝고 서로 다른 종류와 함량의 진세노사이드를 함유하고 있음이 연구되어 왔다. Hybrid 는 PG 와 PQ 의 종간 교잡종 이기 때문에 부모보다 더 높은 생장률과 진세노사이드 함량을 보이는 잡종강세의 특징을 가지고 있으나, 정상적인 화분 발달이 되지 않아 불임의 표현형을 나타낸다. 본 연구에서는 이러한 특성을 가진 Hybrid 개체를 유지하고 불임을 극복하기 위한 방법을 모색하였다. 그래서, 하나의 Hybrid 미성숙 열매로부터 우리는 캘러스, 부정근, 체세포배 그리고 모상근과 같은 다양한 조직들로 성공적으로 유도하였고 각각의 조직을 유도하기 위한 적정 배지 조건을 확립시켰다. 특히 부정근 배양에서 Hybrid 는 PG 와 PQ 보다 각각 2.06 배 와 1.58 배 높은 생장률을 보였다. 또한, Hybrid는 *Agrobacterium rhizogenes* 를 매개한 형질전환을 수행하였을 때 식물생장조절제를 처리한 배지 조건 내에서 형질전환된 모상근이 부정근 보다 약 2.49 배 높은 생장률을 보였으며, 식물생장조절제가 없는 조건에서도 그 생장률이 유지되었다. 이 뿐만 아니라, 핵 그리고 엽록체로부터 유래된 마커들을 사용하여 Hybrid 의 부모종을 명확하게 구별해 내었고, UPLC-QTF/MS 를 통해 Hybrid, PG 그리고 PQ 의 진세노사이드 구성을 비교

함으로써 Hybrid 가 PG 와 PQ 보다 더 많은 45개의 의 진세노사이드를 함유하고 있음을 확인하였다. 결론적으로, 본 연구는 배지에 식물생장조절제들을 달리 처리하여 다양한 조직들을 배양하는 시스템을 확립시켜 우수한 Hybrid 라인을 유지하기 위한 플랫폼을 제공하고자 하였으며, Hybrid 는 높은 생장 속도와 다양하고 많은 함량의 진세노사이드를 함유하고 있다. 따라서 단위 질량당 진세노사이드의 획득량을 높임으로써 앞으로 인삼 산업에서 인삼함유제품의 지표성분인 진세노사이드의 함량 기준치를 보완해주어 품질 향상에 큰 역할을 할 수 있을 것이라고 제안한다.