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Research article

High frequency somatic embryogenesis and plant regeneration of interspecific ginseng hybrid between *Panax ginseng* and *Panax quinquefolius*

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ABSTRACT

Background: Interspecific ginseng hybrid, *Panax ginseng* × *Panax quenquifolius* (*Pgq*) has vigorous growth and produces larger roots than its parents. However, F_1 progenies are complete male sterile. Plant tissue culture technology can circumvent the issue and propagate the hybrid.
Methods: Murashige and Skoog (MS) medium with different concentrations (0, 2, 4, and 6 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D) was used for callus induction and somatic embryogenesis (SE). The embryos, after culturing on GA_3 supplemented medium, were transferred to hormone free $^1/_2$ Schenk and Hildebrandt (SH) medium. The developed taproots with dormant buds were treated with GA_3 to break the bud dormancy, and transferred to soil. Hybrid *Pgq* plants were verified by random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analyses and by LC-IT-TOF-MS.
Results: We conducted a comparative study of somatic embryogenesis (SE) in *Pgq* and its parents, and attempted to establish the soil transfer of *in vitro* propagated *Pgq* tap roots. The *Pgq* explants showed higher rate of embryogenesis (~56% at 2 mg/L 2,4-D concentration) as well as higher number of embryos per explants (~7 at the same 2,4-D concentration) compared to its either parents. The germinated embryos, after culturing on GA_3 supplemented medium, were transferred to hormone free $^1/_2$ SH medium to support the continued growth and kept until nutrient depletion induced senescence (NuDIS) of leaf defoliation occurred (4 months). By that time, thickened tap roots with well-developed lateral roots and dormant buds were obtained. All *Pgq* tap roots pretreated with 20 mg/L GA_3 for at least a week produced new shoots after soil transfer. We selected the discriminatory RAPD and ISSR markers to find the interspecific ginseng hybrid among its parents. The F_1 hybrid (*Pgq*) contained species specific 2 ginsenosides (ginsenoside Rf in *P. ginseng* and pseudoginsenosides F_{11} in *P. quinquefolius*), and higher amount of other ginsenosides than its parents.
Conclusion: Micropropagation of interspecific hybrid ginseng can give an opportunity for continuous production of plants.

1. Introduction

The genus *Panax* (Araliaceae), a perennial deciduous herb, includes 16–18 species of which two grow in the eastern part of North America and the others in Eastern Central Asia [1]. While most species of this genus have medicinal values, only three of them (*Panax ginseng*, *Panax quinquefolius*, and *Panax notoginseng*) have been produced commercially and are highly valued in traditional medicine [2–4].

The medicinal property of ginseng is largely attributed to ginsenoside saponins. Up to now, > 150 naturally occurring ginsenosides have been identified in *Panax* species [5], and while *P. ginseng*

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(*Pg*) is known for containing relatively wider diversity of ginsenoside types, *P. quinquefolius* (*Pq*) is known for its higher content of total ginsenosides compared with *Pg* [6]. Moreover, *Pq* lacks ginsenoside Rf while *Pg* lacks pseudoginsenoside F_{11} and the presence of these compounds in samples is regarded as a litmus test for ginseng adulteration [7,8]. Both *Pg* and *Pq* have antifatigue, antiaging, and antistress effects, but each of them is known to have different pharmacological properties. The higher ratio of protopanaxadiol (PPD)- to protopanaxatriol (PPT)-type ginsenosides in *Pq* as compared to that in *Pg* is regarded as a key factor for such differences [9,10]. Additionally, except for wild ones, *Pq* has Rg1/Rb1 and Rb2/Rb1 ratio of < 0.4 while *Pg* has a ratio of > 0.4 [11].

Despite their differences in the amount and kinds of ginsenosides, both *Pg* and *Pq* are allotetraploid ($2n = 4x = 48$) [12] and are morphologically similar, which has led some researchers to develop their interspecific hybrid (*Pgq*) [6,13–16]. In general, heterosis (hybrid vigor) of F1 progeny in hybrids is exploited in a variety of ways. The aim of plant breeding is to improve plant performance, quality, and yield. *Pgq* interspecific hybrid has higher content of ginsenosides with larger roots and better growth rate in the field than the parents [6,15].

While interspecific hybrid breeding programs offer some hope for generating new plants, fixation of the genetic makeup of F_1 hybrid is cumbersome, and has become more difficult for *Pgq* due to its male sterility [6]. Interestingly, micropropagation has been a useful technique for the clonal propagation of important agronomical and economic plants [17] and it could fix the heterosis obtained in F_1 hybrids. There have been several studies on different aspects of somatic embryogenesis (SE) in *Pg* [18–22] and *Pq* [23–25]. Although fewer in number, SE-related studies have been reported in *Pgq* as well [13,15], however, the practical approach of its clonal micropropagation and successful *ex vitro* transfer to soil have not yet been reported.

In this study, our objective was to optimize the micropropagation procedure for the regeneration of *Pgq* via SE and successfully transplant the regenerants into soil. Additionally, selection of discriminatory random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers as well as comparative analysis of signature compounds (ginsenoside Rf and pseudoginsenoside F11), along with seven common ginsenosides via LC/MS were carried out for the hybrid and its parent lines in the study.

2. Materials and methods

2.1. Plant material collection and culture establishment

Seeds of interspecific hybrid ginseng, *Pgq*, *Pg*, and *Pq* were provided by the National Institute of Horticultural and Herbal Science, Soi-myeon 170-3, Eumseong-gun, Chungcheongbuk-do 24341, Korea. *Pg* had been used as maternal parent (Chunpoong cultivar) and *Pq* (collected from Wisconsin, USA) as a pollen donor. Interspecific hybrids were produced by artificial crosspollination of mature pollen of *Pq* onto the stigmas of maternal *Pg* plants. The pollinated flowers were capped with paper bags to prevent undesired pollination. The seeds of the interspecific hybrid, *Pgq*, were harvested from mature fruits. Leaf petioles collected from germinated plants of each genotype were immersed in 70% (v/v) ethanol for 30 s and in 2% sodium hypochlorite for about 20 min, followed by three washes with sterile water for 2 min each. The petioles were left to dry under the hood for next 30 min, after which, they were cut in to ~0.5-mm pieces and cultured in Petri dishes containing Murashige and Skoog (MS) [26] medium supplemented with 3% sucrose and different concentrations (0, 2 mg/L, 4 mg/L, and 6 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D) to access their callus-producing and embryogenic properties. The explants were kept at 24±2°C in dark for a month to induce callus. Twenty explants were used per treatment, and each treatment was repeated three times. After callus induction, the cultured Petri dishes were transferred onto 16/8 h (light/dark) photoperiod with white fluorescent light (80 μmol/m^2/s) at 24 ± 2°C. The data on callus induction, embryogenesis, and embryos per explant were collected at the end of 8 wk of culture.

2.2. Embryo handling and soil transfer

Embryo maturation and hardening followed by subsequent soil transfer and acclimatization of the plantlets were carried out as described by Kim et al [27], with modification. The somatic embryos at the cotyledonary stage were transferred to SH medium [28] supplemented with 5 mg/L gibberellic acid (GA_3) for germination. After 2 wk, the green and elongated plantlets were separated and transferred to hormone-free $^1/_2$ SH medium in polycarbonate magenta culture boxes and kept in them without further medium substitution until leaf senescence was induced by nutrient depletion. Before the plantlets were transferred to soil, we tested the effect of GA_3 on bud dormancy breakage. The taproots with thickened crown region were subjected to GA_3 treatment for different durations (0, 1 wk, 2 wk, and 3 wk). Soil was prepared by mixing horticulture grade soil and perlite at 3:1 ratio, pH was adjusted to 7.0, and put in plastic square boxes (25 cm × 20 cm × 15 cm) followed by sterilization. Twenty roots were planted per box.

2.3. Hybrid verification by RAPD and ISSR analysis

Fresh leaves of the samples (*Pg*, *Pq*, and *Pgq*) were collected and cut to extract genomic DNA. After freezing with liquid nitrogen, the samples were ground into fine powders using a mortar and pestle. Total genomic DNA was extracted using DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA). DNA concentrations of 3 samples were measured with an UV spectrophotometer (Shimazu Co., Japan). Polymerase chain reaction was performed using the method of Ahn et al [29], with modification. We selected RAPD and ISSR primers, three each, obtained from The University of British Columbia, Vancouver, Canada (Tables 1 and 2). Polymerase chain reaction amplification was run on a DNA thermal cycler (Applied Biosystems, Biopolis, Singapore). Cycling conditions for RAPD analysis were 94°C for 5 min; followed by 40 cycles of denaturation at 94°C for 1 min, annealing temperature at 55°C for 1 min, polymerization at 72°C for 1 min, and final extension at 72°C for 7 min. For ISSR, the first heating temperature was at 94°C for 5 min; followed by 45 reaction cycles of 30 s at 94°C, 60 s at 52°C, 60 s at 72°C, and a final 10 min at 72°C. The reaction samples were loaded on to 1% agarose gel and analyzed for polymorphism pattern of each primer.

2.4. Saponin analysis

To analyze ginsenoside contents among *Pg*, *Pq*, and *Pgq*, roots of *in vitro* raised plants were sampled and dehydrated at 50°C in a

Table 1
Primers used and polymorphisms shown by them in RAPD analysis of *Pgq* and its parents

Primer ID	Sequence (5′–3′)	Total amplicons	Polymorphic amplicons	Polymorphism (%)
UBC 536	GCC CCT CGT C	10	2	20.0
UBC 540	CGG ACC GCG T	13	3	23.08
UBC 594	AGG AGC TGG C	13	4	30.77
Total amplicons		36	9	25.0

Pgq, *Panax ginseng Panax quinquefolius* hybrid; RAPD, random amplified polymorphic DNA

Table 2
Primers used and polymorphisms shown by them in ISSR analysis of *Pgq* and its parents

Primer ID	Sequence (5′–3′)	Total amplicons	Polymorphic amplicons	Polymorphism (%)
UBC 809	AGA GAG AGA GAG AGA GG	7	2	28.57
UBC 818	CAC ACA CAC ACA CAC AG	7	3	42.85
UBC 827	ACA CAC ACA CAC ACA CG	10	3	30
Total amplicons		24	8	33.33

ISSR, inter simple sequence repeat; *Pgq*, *Panax ginseng Panax quinquefolius* hybrid

drying oven for 3 h. Each sample was ground in a mortar and the milled powder was soaked in 80% methanol, followed by sonication for 30 min. After centrifugation of solutes, the supernatants were filtered using a SepPak C-18 Cartridge (Waters, Milford, MA, USA). LC experiments were conducted on a Shimadzu HPLC system equipped with LC-20AD binary pump, DGU-20A degasser, SIL-20A autosampler, CTO-20AC column oven, and SPD-M20A PDA detector. The mobile phase (delivered at 0.5 mL/min) comprised Solvent A (water) and Solvent B (acetonitrile). A binary gradient elution was performed for ginsenoside analysis: initial 15% B from 0 to 0.5 min, linear gradient 15–25% B from 0.5 min to 5 min, linear gradient 25–30% B from 5 min to 13 min, linear gradient 30–35% B from 13 min to 20 min, linear gradient 35–40% B from 20 min to 25 min, linear gradient 40–100% B from 25 min to 30 min, linear gradient 100–75% B from 30 min to 35 min linear gradient 75–15% B from 35 min to 40 min, and a final quick return to the initial 15% B, which was maintained until 40 min to balance the column. The flow rate was set to 0.2 mL/min and sample injection volume was 5 μL.

Chromatographic separation was achieved on a YMC-Pack Pro C18 RS column (5μm, 2.0mm × 150mm; YMC, Japan) at 40°C. LC ion trap time-of-flight mass spectrometry (LC-IT-TOF-MS) (Shimadzu) equipped with an atmospheric-pressure chemical ionization (APCI) source was used in positive ion mode. The following optimized analytical conditions were used: detector voltage, 1.60 kV; nebulizing gas (N_2) flow, 1.5 L/min; dry gas (N_2) flow, 50 kPa; pressure of TOF region, 1.5×10^{-4} Pa; ion trap pressure, 1.7×10^{-2} Pa; ion accumulation time, 30 ms; and precursor ion selected width, 3.0 amu. For the qualitative analysis, the scan ranges were set at m/z 100–1,000 for MS^1, 100–1,000 for MS^2; ultrahigh purity argon was used as the cooling and collision gas for the collision-induced dissociation (CID) experiments, and the collision energy was set at 50% for MS^2. The standard compounds of ginsenoside Rf, pseudoginsenoside F_{11}, and other common typical ginsenosides (ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1) for LC-IT-TOF-MS analysis were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.5. Statistical analysis

The percentage data of callus induction and embryogenesis were arcsine transformed and the significance of the effect of treatment was tested by analysis of variance (ANOVA) on the statistical programming environment R (version 3.1.1, worldwide R-core team). Normality was evaluated by Shapiro–Wilk normality test, and the nonparametric Kruskal–Wallis test was applied for data of the mean number of somatic embryos. Mean values were calculated with standard errors. Multiple comparisons were made by using Tukey's honest significant difference at a significant level of 5%.

3. Results

3.1. Callus induction, embryogenesis, and somatic embryo production

Petioles of all three ginseng plants (*Pg*, *Pq*, and *Pgq*) were cultured on MS medium supplemented with different concentrations of 2,4-D. In most cases, explants were swollen after 2 wk. Yellow–green calli were formed after 4 wk of culture, and somatic embryos were produced on the surface of the callused explants after 8 wk (Fig. 1A). Callus induction was observed in most explants on MS medium with different levels of 2,4-D (Fig. 2A). Although the explants from all three explants showed the lowest response on hormone-free MS medium, the *Pgq* explants showed somatic embryogenesis at a low rate, even in hormone-free medium. ANOVA indicated that 2,4-D concentration significantly affected callus induction ($p < 0.001$, $F = 153.37$, $df = 3$). A significant difference was also observed in callus induction among three explant sources ($p < 0.012$, $F = 5.65$, $df = 2$). Among the three, *Pq* showed the lowest frequency of callus induction. The frequency of the embryogenesis also differed significantly according to concentration of 2,4-D ($p < 0.001$, $F < 44.42$, $df = 3$). Interestingly, unlike the frequency of callus induction, the highest frequency of embryogenesis was obtained in *Pgq*. Even though 2,4-D concentrations from 2 mg/L to 6 mg/L did not show a significant difference in the frequency of embryogenesis in *Pgq*, the highest frequency of embryogenesis was observed at 2 mg/L 2,4-D (56.5%; Fig. 2B). Many somatic embryos at different development stages were observed protruding from the explants (Fig. 1A). The number of somatic embryos per explant was significantly different among the explant sources and 2,4-D treatments ($p < 0.001$, $\chi^2 = 64.267$, $df = 2$ and $p < 0.001$, $\chi^2 = 83.298$, $df = 3$, respectively; Fig. 2C). Moreover, ANOVA indicated a significant interaction between ginseng species and 2,4-D concentrations ($p < 0.001$, $F = 5.65$, $df = 6$). This was probably due to the fact that *Pgq* explants produced higher numbers of somatic embryos on MS medium supplemented with 2 mg/L 2,4-D as compared to any other explant source × hormone combinations.

3.2. Germination and plantlet conversion

The somatic embryos on SH medium containing 5 mg/L GA_3 germinated rapidly within 2 wk (Fig. 1B). After transfer onto $^1/_2$ SH medium, germinated embryos developed into plantlets with both shoots and roots (Fig. 1C). Initially, the plantlets had single and slender primary roots but by the end of 2 mo of culture, they were distinctively bifurcated and crown regions of the taproots started thickening (Fig. 1D). After prolonged culture for 4 mo, plants had taproots with well-developed lateral roots but completely senesced their leaves. At the time, thickness of the roots was the largest at crown region, which showed protruding but unbroken buds on its surface (Fig. 1E). The taproots were precultured in $^1/_2$ SH medium supplemented with 20 mg/L GA_3 for 3 wk, and transferred to the soil. Leaves of newly sprouted buds were observed coming from the transferred roots on the soil surface at 2 wk after transfer (Fig. 3A) and 3 wk after transfer (Fig. 3B). No significant difference was observed in germination among the roots under different durations of GA_3 treatment (data not shown). However, the taproots without GA_3 treatment did not show any sign of germination (data not shown) (Fig. 3C). Soil transferred roots with newly grown shoots had freshly developed new roots after digging up the plant (Fig. 3D). The sprouts were fully expanded at 2 mo after soil transfer

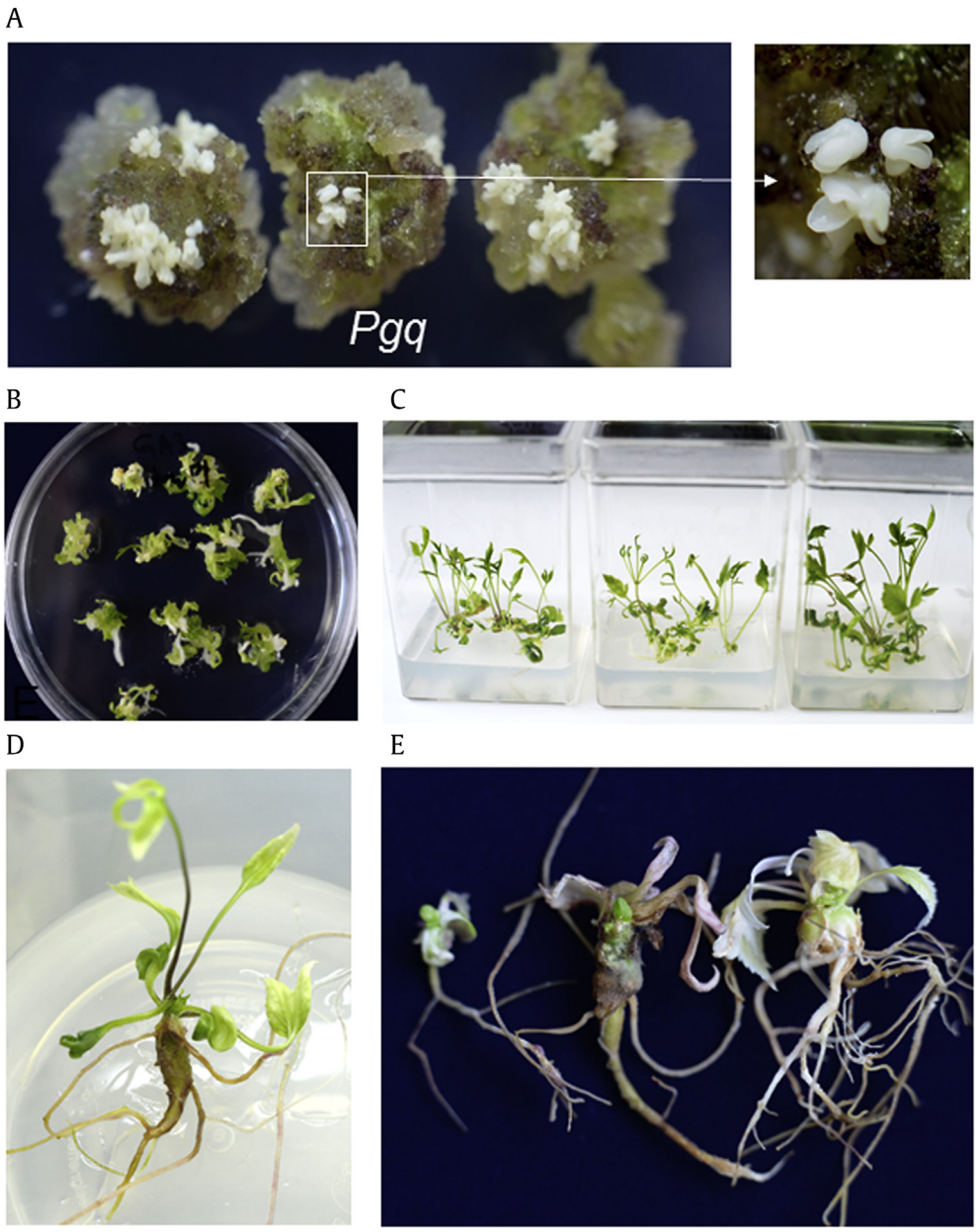

Fig. 1. Callus induction, somatic embryo production and plant regeneration of interspecific hybrid ginseng (*Pgq*). (A) *Pgq* explants on Murashige and Skoog [26] medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid producing somatic embryos after 8 wk of culture. Arrow indicates individual somatic embryos at cotyledonary stage. (B) Germinating somatic embryos on Schenk and Hildebrandt (SH) medium supplemented with 5 mg/L gibberellic acid. (C) Plantlets with shoots and roots on $^{1}/_{2}$ SH medium in magenta boxes. (D) Plantlet with thickened root near the crown after 3 mo of culture. (E) Thickened taproots after leaf senescence due to prolonged culture for 4 mo. Arrows indicate the new dormant buds. *Pgq*, *Panax ginseng Panax quinquefolius* hybrid.

(Fig. 3E). Although we found fungal contamination on the surface of soil in plastic square boxes, > 80% of plantlets survived (data not shown).

3.3. RAPD and ISSR analysis for discriminative identification of Pgq and parent lines

To distinguish *Pgq* from *Pg* and *Pq*, primers of random RAPD and ISSR markers were tested, among which, three each for RAPD and ISSR showed polymorphisms (Tables 1 and 2, Figs. 4A and 4B). The size of the amplicons produced by the primers ranged from 200 bp to 8,000 bp. The highest polymorphism obtained for ISSR was 42.85% with UBC 818 primer (Table 2), while that for RAPD was 30.77% with UBC 594 primer (Table 1). Total amplification of ISSR and RAPD reaction products were 24 and 36, showing 33% and 25% polymorphisms respectively (Tables 1 and 2).

The primers produced some RAPD and ISSR amplicon bands of different sizes for the parents, while the *Pgq* shared all or some bands from them, indicating a typical interspecific hybrid polymorphism (Figs. 4A and 4B). Amplified bands using primers (UBC 809, 818, and 827) in ISSR analysis clearly showed common bands for the parents (Fig. 4B). In RAPD analysis, UBC 536 and UBC 594 primers discriminatively identified *Pgq* from its parents. Compared to *Pg*, *Pq* showed more polymorphic bands with UBC 540 and 594 (Figs. 4B and 4C). UBC 540 produced unique RAPD products for *Pq* that were shared by *Pgq* but no such band was observed for *Pg*

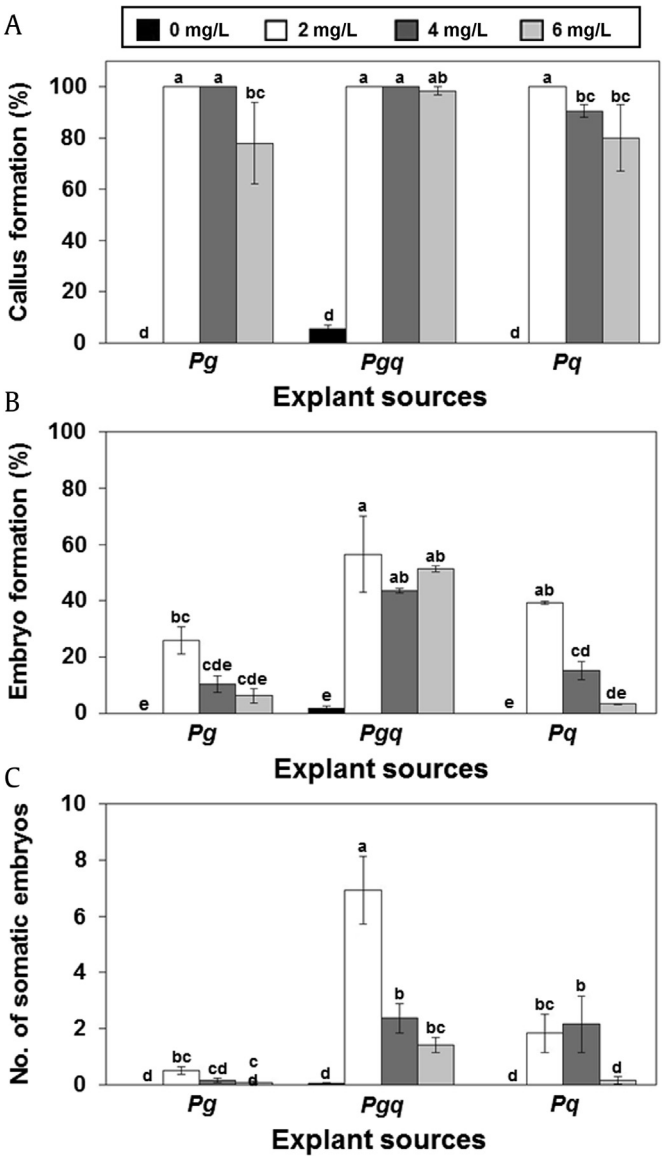

Fig. 2. Effect of 2,4-dichlorophenoxyacetic acid concentrations on callus induction, embryogenesis, and somatic embryo production. (A) Frequency of callus induction. (B) Frequency of embryogenesis. (C) Mean number of somatic embryos. Data are presented as mean ± standard error. Columns with the same letter show no significant difference according to Tukey's honest significant difference test at $p \leq 0.05$.

(Fig. 4A, blue arrows). Compared to RAPD primers, ISSR primers showed better discrimination for *Pgq* hybrid identification as its amplified bands had shared clear bands (Fig. 4B).

3.4. Comparative analysis of ginsenosides

We performed a comparative analysis of ginsenoside profiles by LC-IT-TOF-MS, and particularly focused on the occurrence of both ginsenosides Rf and pseudoginsenoside F_{11} in *Pgq*. Our results clearly showed the presence of both ginsenosides Rf and pseudoginsenoside F_{11} in addition to six other major compounds (Rb1, Rb1, Rc, Rd, Re, and Rg1) in *Pgq*. However, pseudoginsenoside F_{11} was not detected in parental *Pg*, and ginsegnside Rf was not detected in *Pq* (Fig. 5). Both pseudoginsenoside F_{11} and ginsenosides Rf had the same molecular weight (800.27, $C_{42}H_{72}O_{14}$). Both the pseudoginsenoside F_{11} and ginsenosides Rf were detected as a single peak by UV mode (Fig. 5D). However, the chromatogram of total ion chromatogram (TIC) MS analysis resulted in two separated peaks to distinguish the pseudoginsenoside F_{11} and ginsenosides Rf (Fig. 5E). Mass fragments resulting from positive ionization were clearly different because of the difference in molecular structures of the compounds (pseudoginsenoside F_{11} and ginsenosides Rf) (Fig. 6). LC-IT-TOF-MS analysis detected ginsenoside Rf (16.0 min) and pseudogensenoside F11 (16.9 min) in *Pg* and *Pq* respectively, while both compounds were detected in *Pgq* at the aforementioned retention times (Fig. 5). The measurement of accurate MS analysis using positive ionization mode for pseudoginsenoside F_{11} revealed $[M+H]^+ = 801.2704$, the typical consecutive loss of water molecules: $[M+H\text{-}(\text{-Glc-Rha})\text{-}H_2O]^+ = 475.2410$, $[M+H\text{-}(\text{-Glc-Rha})\text{-}2H_2O]^+ = 457.2384$, $[M+H\text{-}(\text{-Glc-Rha})\text{-}3H_2O]^+ = 439.2333$, $[M+H\text{-}(\text{-Glc-Rha})\text{-}4H_2O]^+ = 421.2291$, and $[M+H\text{-}(\text{-Glc-Rha})\text{-}5H_2O]^+ = 403.2181$. Similarly, ginsenoside Rf revealed $[M+H\text{-}2H_2O]^+ = 765.2522$, and the typical consecutive loss of water molecules: $[M+H\text{-}(\text{-Glc-Glc})\text{-}2H_2O]^+ = 441.2366$, $[M+H\text{-}(\text{-Glc-Glc})\text{-}3H_2O]^+ = 423.2430$, $[M+H\text{-}(\text{-Glc-Glc})\text{-}4H_2O]^+ = 405.2453$ (Fig. 6). These mass fractions for pseudoginsenoside F_{11} and ginsenosides Rf detected in *Pgq* were exactly matched with those of authentic standards (pseudoginsenoside F_{11} and ginsenoside Rf compounds). The results clearly revealed that the roots of *Pgq* contained *Pg*-specific ginsenoside Rf and *Pq*-specific pseudoginsenoside F_{11}.

To compare the ginsenoside content in roots of *Pg*, *Pq*, and *Pgq*, six typical ginsenosides (Rb1, Rb2, Rc, Rd, Re, and Rg1), along with the two species-specific compounds (ginsenoside Rf and pseudoginsenoside F_{11}), were measured by comparison of authentic standard ginsenosides (Fig. 7). Generally, the roots of *Pgq* contained higher amounts of ginsenosides compared to its parents, except for ginsenosides Rb2 and Rf and pseudoginsenoside F_{11}.

4. Discussion

Ginseng plants are recalcitrant to embryogenesis and regeneration *in vitro* [25,30,31]. There have been several studies of micropropagation via SE systems in ginseng species [32–34]. According to Lim et al [35], petioles are the best material for callus induction. Our study also showed the petioles as appropriate material for callus induction. In plant tissue culture, plant growth regulators are indispensable and essential to the process of SE. In particular, 2,4-D is the most frequently used (49%) among auxins [36]. In general, it has often been used to induce somatic embryos [37–39]. Earlier Choi [40] reported 2 mg/L 2,4-D as the optimum concentration for callus induction in *Pg*, contradictory to which, our study did not find any significant difference in callus induction between 2 mg/L and 4 mg/L 2,4-D treatment. However, 2 mg/L 2,4-D was an appropriate concentration for somatic embryo production. Similar results have also been reported by Chang and Hsing [18] and Choi et al [41]. Higher auxin concentrations for longer duration have been reported to inhibit somatic embryo development past the globular stage in carrot somatic embryos cocultured with *Arabidopsis thaliana* cells [42]. Wernicke and Milkovits [43] also reported the inhibitory effect of high 2,4-D concentration (30 mg/L) on normal cell differentiation and morphogenesis of wheat meristems. Interestingly, our study showed that 2 mg/L 2,4-D could be the optimum concentration for somatic embryo production in *Pgq* and either of its parents, even though the performance of the parents was comparatively low. Although both *Pg* and *Pq* are highly self-pollinated, the lower performance of their embryogenesis could be attributed to their inbreeding depression. This has already been documented in *Pq* [44]. This might have been surpassed by their hybrid *Pgq* due to heterosis or, more appropriately, heterobeltiosis. The phenomenon is frequently observed in hybrid plants and animals [45,46].

Fig. 3. Soil transfer of thickened roots and sprouting of new leaves. (A, B) Young sprouting on the surfaces of soil at 3 wk after transfer of thickened roots in a polypropylene container containing sterilized soil. (C) Soil-transferred thickened roots of *Pgq* are successfully producing shoots until before fully expanding its leaves. (D) A dug up plant showing freshly developed new roots (arrows) and shoots at 1 mo after soil transfer. (E) Soil-transferred *Pgq* roots with fully expanded leaves at 2 mo after soil transfer. *Pgq*, *Panax ginseng* *Panax quinquefolius* hybrid.

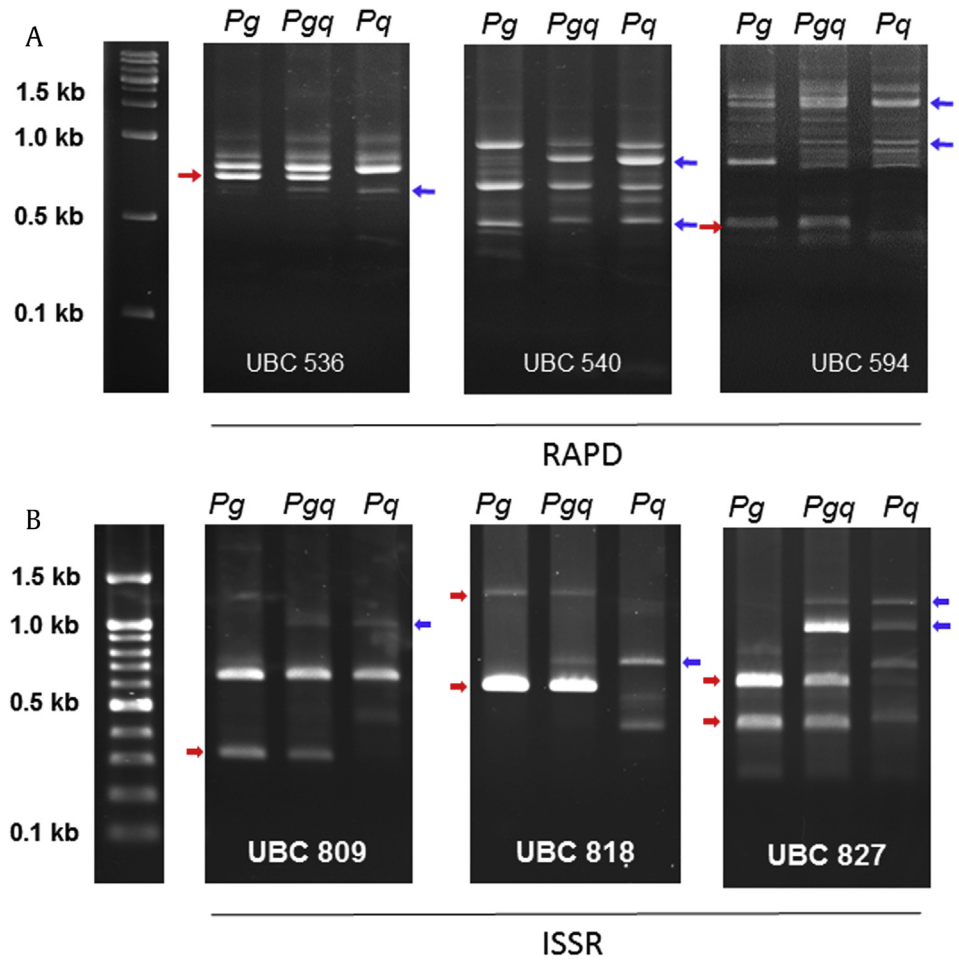

Fig. 4. DNA profiles of *Pg*, *Pgq*, and *Pq* with different polymorphic RAPD and ISSR markers. (A) UBC 536, UBC 540, and UBC 594 RAPD markers. (B) UBC 809, UBC 818, and UBC 827 ISSR markers. *Pg* and *Pq* specific amplicons are indicated by red and blue arrows, respectively. ISSR, inter simple sequence repeat; *Pg*, *Panax ginseng*; *Pgq*, *Panax ginseng Panax quinquefolius* hybrid; *Pq*, *Panax quinquefolius*; RAPD, random amplified polymorphic DNA.

Soil transfer of newly regenerated ginseng plantlets is difficult because they are weak, with poorly developed root systems and susceptibility to fungal infection after soil transfer. We have established a viable approach of hardening the embryo-derived plantlets followed by their successful soil transfer. Although there are some reports of soil transfer of *in vitro* plantlets of *Pg* [20,30,41,47] and *Pq* [23–25], there has not been any report of successful transfer of their hybrid *Pgq*. In our study, the SE-derived *Pgq* plantlets were continuously grown in the same medium, leading the plant to senesce its leaves due to nutrient depletion. Such nutrient-depletion-induced senescence (NuDIS) has been reported in *Arabidopsis* [48], and a similar practice for *in vitro* hardening has been reported in gladioli corms [49]. Since root is the major sink of carbon resource storage in ginseng [50], the defoliated ginseng roots might have been hardened and nutrient rich. The NuDIS-derived hardening is due to the increased content of endogenous abscisic acid (ABA) [51]. The increased ABA simultaneously causes dormancy in buds developing in the root crown, thereby hindering its immediate sprouting upon soil transfer [27]. Interestingly, GA_3 could be used to surpass plant bud dormancy [52], which has been practiced in ginseng as well [15,20,25,53]. Our study showed that 20 mg/L GA_3 treatment of NuDIS-derived *Pgq* taproots for at least 1 wk is sufficient to break bud dormancy. This agrees with the finding of a recent study on *Pg* that showed that 10–40 mg/L GA_3 broke its bud dormancy [27].

Detection of molecular markers is an efficient method to distinguish the hybrid from its parents. RAPD has been used for this purpose in both plant and animal systems [54,55]. RAPD and restriction fragment length polymorphism analyses have similar ability to show sequence polymorphism, but the former is preferred due to rapidity of analysis and ability to detect *de novo* polymorphism [54]. Our study showed that the six selected primers for RAPD and ISSR analysis (3 each) detected polymorphism for *Pgq* and its parents, thereby discriminating one from the other. Due to such a feature, these markers have been used to determine cross-pollinated and self-pollinated progenies in alfalfa [56]; to screen somatic hybrids of *Solanum tuberosum* and *Solanum brevidens* [57]; to detect quantitative trait loci in interspecific hybrid progeny of *Eucalyptus* [58] and blue grass [59]; to identify hybrids in rice [60]; and to test molecular genetic diversity of cotton Mehr cultivar and its cross-progenies [61]. However, as we observed in the present study, ISSR primers are preferred over RAPD to detect polymorphism [62].

Pq lacks ginsenoside Rf and *Pg* lacks pseudoginsenoside F_{11}, thus the presence of the two in a sample is regarded as important evidence of ginseng adulteration [7,8]. Although earlier findings have shown the ginsenoside profiles in *Pgq* by [6,15,34], they did not detect the key compounds (ginsenoside Rf and pseudoginsenoside F_{11}) in F1 hybrid (*Pgq*). In our present work, LC-IT-TOF-MS analysis clearly demonstrated the presence of both compounds in the roots

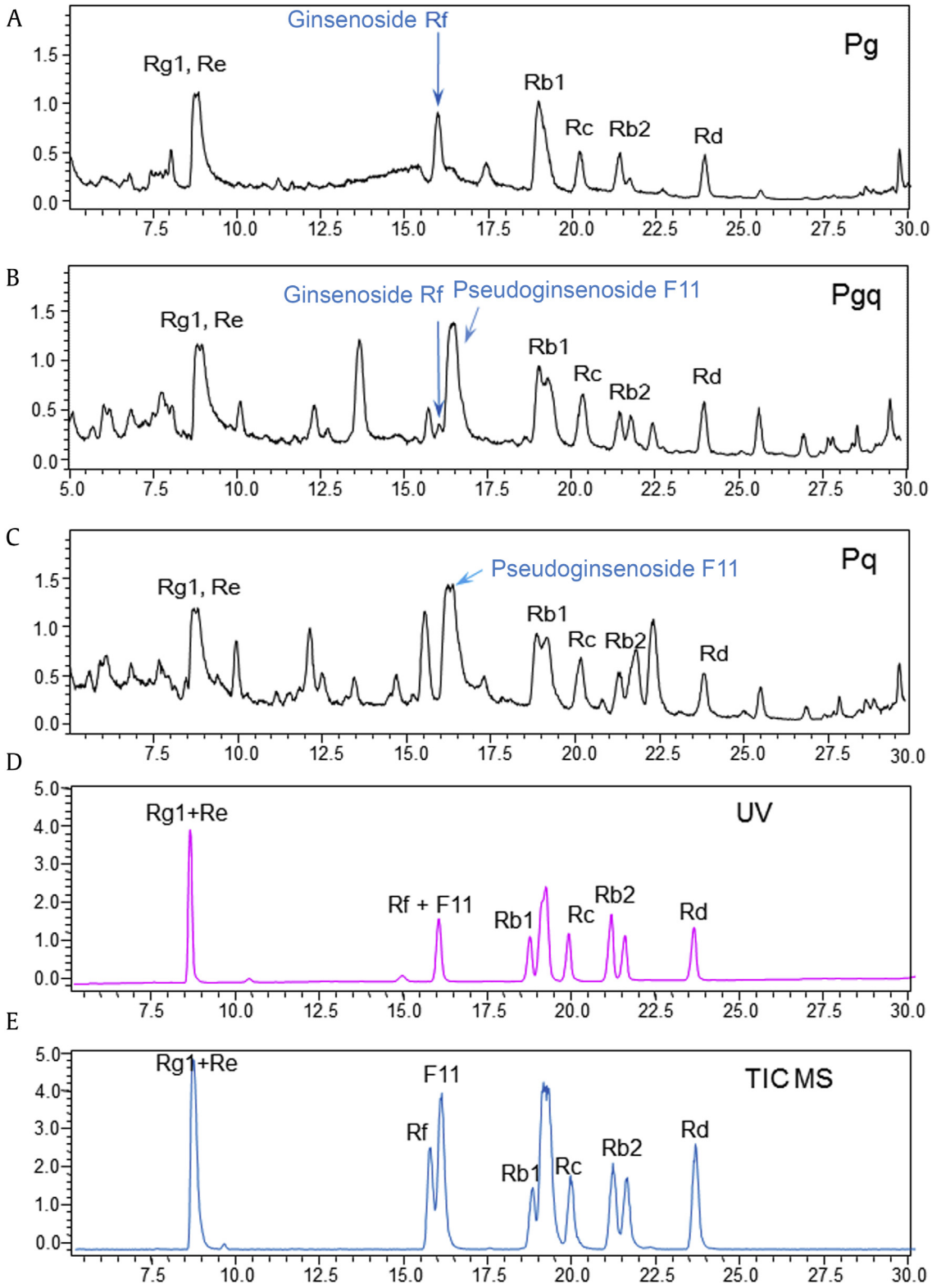

Fig. 5. Total ion chromatogram (TIC) of extracts of *Pg*, *Pgq*, and *Pq* by LC-IT-TOF-MS analysis. Ionized peaks in three samples (*Pg*, *Pq*, and *Pgq*) reveals that ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc, and Rd are common in all samples (A–C) but ginsenoside Rf is clearly seen in both *Pg* (A) and *Pgq* (B), and pseudogensenoside F_{11} is clearly seen in *Pq* (C) and *Pgq* (B). The ginsenoside Rf and pseudogensenoside F_{11} in samples are perfectly matched with standard TICs (D). LC-IT-TOF-MS, LC ion trap time-of-flight mass spectrometry; *Pg*, *Panax ginseng*; *Pgq*, *Panax ginseng Panax quinquefolius* hybrid; *Pq*, *Panax quinquefolius*.

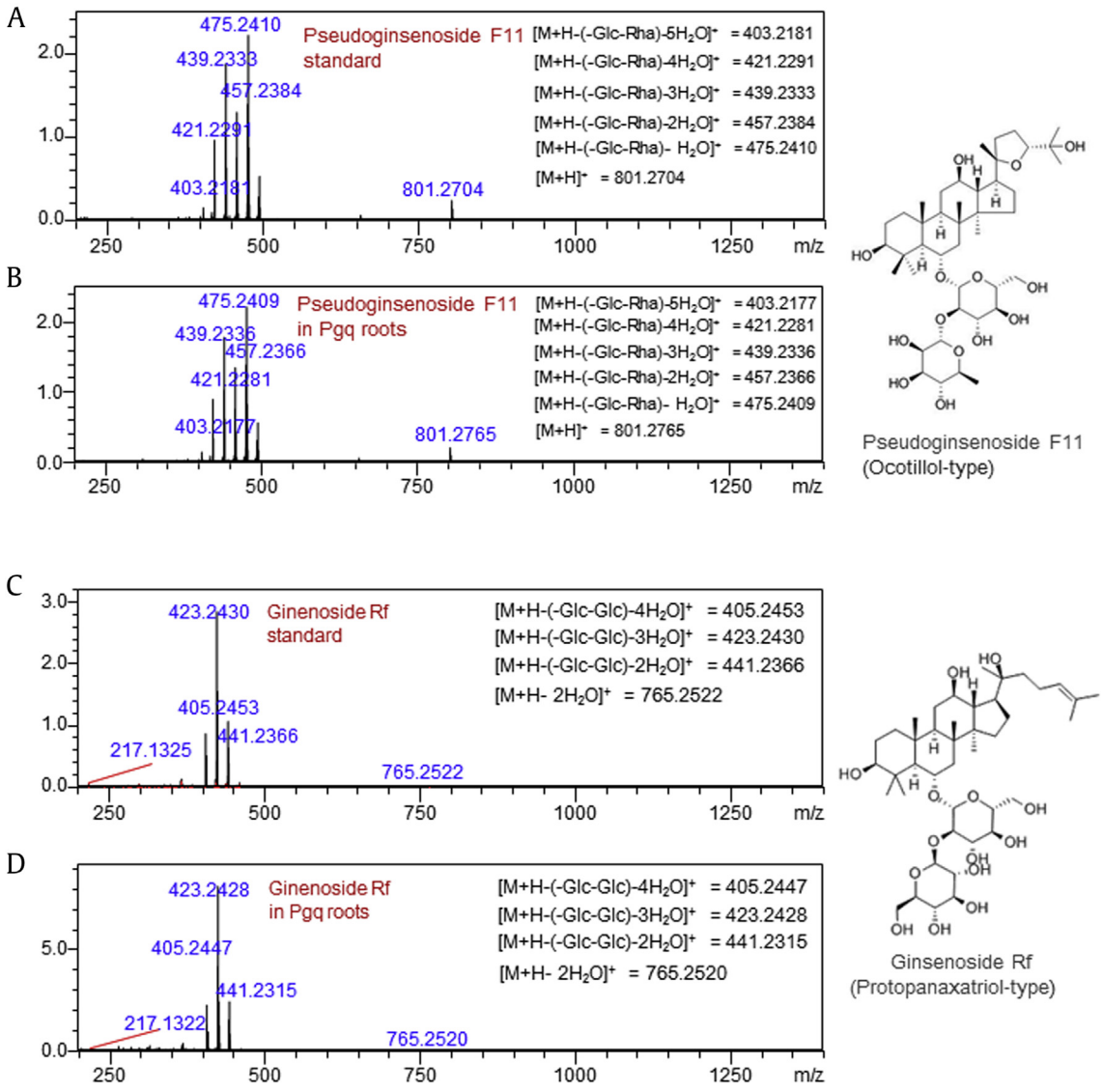

Fig. 6. Mass spectra of ginsenoside Rf and pseudogensenoside F_{11} of standards (A and C) and extracted saponins (B and D) of *Pgq* in positive ion mode. Structures of pseudoginsenoside F_{11} octillol-type skeleton and ginsenoside F_1 protopanaxatriol-type skeleton are shown adjacent to their respective mass spectra. *Pgq*, *Panax ginseng Panax quinquefolius* hybrid.

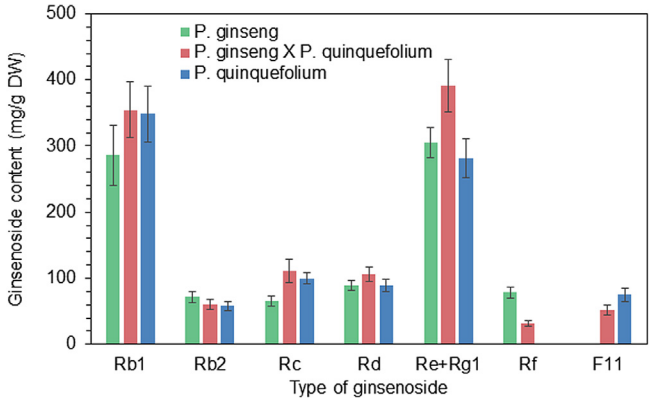

Fig. 7. Ginsenoside contents in the roots of *in vitro* cultured plantlets of *Pg*, *Pgq*, and *Pq*. Data are presented as mean ± standard error ($n = 3$ each). *Pg*, *Panax ginseng*; *Pgq*, *Panax ginseng Panax quinquefolius* hybrid; *Pq*, *Panax quinquefolius*.

of *Pgq* hybrid. This result establishes the fact that F1 hybrid produces all parent-specific ginsenosides, including the unique signature compounds.

Comparative analysis of ginsenoside content among *Pg*, *Pq*, and *Pgq* showed that the roots of *Pgq* contained a higher ginsenoside content compared to its parents, although the amount of each ginsenoside was not clearly higher than in its parents. This indicates that *Pgq* has advantages for both content and diversity of ginsenosides compared to its parents.

Now, we established the mass propagation of interspecific hyrbrid ginseng via somatic embryogenesis. Propagation of interspecific hybrid ginseng should rely on micropropagation because of its male sterility and possible segregation upon backcross with one of its parents. The practice can be extended to its parents since it takes about 18–22 mo of seed maturation for *Pq* and *Pg* in the wild [20,63,64]. Additionally, we demonstrated that the interspecific hybrid ginseng (*Pgq*) not only shared the common parent-specific RAPD and ISSR markers, but also the parent-specific signature compounds (ginsenoside Rf and pseudoginsenoside F_{11}). These

results can be used to distinguish interspecific *Pgq* hybrid in a mixed population of ginseng.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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