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Development of an ISSR-Derived SCAR Marker in Korean Ginseng Cultivars (*Panax ginseng* C. A. Meyer)

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Recently, new ginseng cultivars having superior agricultural traits have been developed in Korea. For newly developed plant cultivars, the identification of distinctiveness is very important factors not only in plant cultivar management but also in breeding programs. Thus, eighty-five inter simple sequence repeat (ISSR) primers were applied to detect polymorphisms among six major Korean ginseng cultivars and two foreign ginsengs. A total of 197 polymorphic bands with an average 5.8 polymorphic bands and 2.9 banding patterns per assay unit across six Korean ginseng cultivars and foreign ginsengs from 236 amplified ISSR loci with an average 6.9 loci per assay unit were generated by 34 out of 85 ISSR primers. Three species of *Panax ginseng* including the Korean ginseng cultivars, *P. quinquefolius,* and *P. notoginseng,* could be readily discriminated using most tested primers. UBC-821, UBC-868, and UBC-878 generated polymorphic bands among the six Korean ginseng cultivars, and could distinguish them from foreign ginsengs. Sequence characterized amplified region (SCAR) marker system was introduced in order to increase the reproducibility of the polymorphism. One SCAR marker, PgI821C650, was successfully converted from the randomly amplified polymorphism by UBC-821. It showed the expected dominant polymorphism among ginseng samples. In addition, the specific polymorphism for Sunwon was generated by treating *Taq* I restriction enzyme to polymerase chain reaction products of PgI821C650. These results will serve as useful DNA markers for identification of Korean ginseng, especially Sunwon cultivar, seed management, and molecular breeding program supplemented with marker-assisted selection.

Keywords: *Panax ginseng*, Inter simple sequence repeat, Korean ginseng, Sequence characterized amplified region

INTRODUCTION

The genus *Panax* (Araliaceae family) consists of more than 10 species which are most famous and important medicinal plants [1]. Among them, ginseng (*P. ginseng* C. A. Meyer) is a most valuable medicinal root crop. It has been utilized for over 2,000 years in Oriental countries as a tonic, a stimulant, and a fatigue-resistance medicine. Regarding human health, many biologically active effects of ginseng has been reported such as immune system stimulation [2], anti-carcinogenic activity [3,4], reduction of blood glucose levels [5], anti-hyperlipidemic ef-

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fects [6].

The majority of commercially available ginseng is cultivated in Northeast Asian countries such as Korea, China, and Japan [7,8]. Particularly, Korean ginseng has globally high demand of consumers due to traditionally accumulated cultivation techniques, as well as the unique processing skills for the manufacture of red ginseng [9]. In addition, new Korean ginseng cultivars, which have superior agricultural traits such as high yield, resistance to disease, and high content of functional secondary metabolites, have been developed for last several decades [10-12]. In recent years, however, Korean ginseng has been frequently produced and illegally distributed by blending with other *Panax* species, such as *P. quinquefolius and P. notoginseng,* because of its relatively higher value in medicinal herb market. Furthermore, discrimination of domestic Korean ginseng cultivars is very difficult owing to their similar morphology when they are mixed with each. These problems are inhibiting the development of the Korea's ginseng industry. Thus, the demand of scientific evidences has increased for discriminating either Korean ginseng from other species or between Korean ginseng cultivars.

In order to solve such problems, molecular identification techniques using DNA marker have been recently applied rather than a traditional discrimination method that is carried out via comparisons of morphological traits.

With inter simple sequence repeat (ISSR), which is now one of the most popular molecular techniques, many studies have been extensively conducted for population genetics, species discrimination, cultivar identification, genetic diversity, and relationship estimation studies [13-16]. This technique requires neither genomic DNA sequence information nor large amounts of DNA, and allows rapid and easy identification [17]. However, the ISSR technique is quite limited due to low reproducibility, because they are sensitive to various experimental conditions. In order to improve its reproducibility, the sequence characterized amplified region (SCAR) marker system was introduced. It is the polymerase chain reaction (PCR) amplification of genomic DNA fragments with specific primers defined from the random amplified polymorphic DNA and ISSR polymorphism sequences. The SCAR marker technique has been successfully applied in many crops for the marking of resistance genes and marker-assisted selection [18-20].

Therefore, this study was conducted to develop the easier discrimination method, which could demonstrate genetic polymorphisms among the Korean ginseng cultivars, *P. quinquefolius,* and *P. notoginseng* by using ISSR marker technique and conversion of polymorphic ISSR markers to SCAR markers.

MATERIALS AND METHODS

Plant materials

Six Korean ginseng cultivars, 'Chunpoong', 'Yunpoong', 'Gopoong', 'Kumpoong', 'Sunpoong', 'Sunwon', and two foreign ginseng, *P. quinquefolius* and *P. notoginseng,* were used for ISSR analysis. These plant materials were preserved and cultivated at the experimental field of National Institute of Horticultural and Herbal Science (NIHHS) of Rural Development Administration, Chungbuk Province, Korea, and voucher samples were deposited at Korea medicinal herbarium at NIHHS (Table 1).

DNA extraction

Total genomic DNAs were extracted from fresh leaves of 3 year old plants of each cultivars and foreign ginseng by using Dneasy Plant Mini Kit (Qiagen, Hilden, Germany). The concentration of DNA was then determined based on a comparison of the plant DNA samples with commercial standard lambda DNA on 1% (w/v) agarose gel, after which it was adjusted to 5 ng/μL.

Table 1. Korean ginseng cultivars and *Panax* species used in this study

No.	Cultivars or species	Voucher no.	Remarks[1)]
1	*P. ginseng* cv. Chunpoong	MPS002375	For red ginseng
2	*P. ginseng* cv. Yunpoong	MPS002380	For white ginseng; high-yielding
3	*P. ginseng* cv. Gopoong	MPS002385	High content of saponin
4	*P. ginseng* cv. Kumpoong	MPS002390	Resistant to disease
5	*P. ginseng* cv. Sunpoong	MPS002395	High-yielding
6	*P. ginseng* cv. Sunwon	MPS002400	For red ginseng
7	*P. quinquefolius*	MPS002405	Heat tolerance
8	*P. notoginseng*	MPS002410	Chinese traditional medicine

[1)] Kim *et al*. [22]

PCR amplification with ISSR primers

PCR primers for ISSR analysis were obtained from University of British Columbia, Canada. ISSR PCR was performed in total 30 μL reaction volume containing 10 ng of DNA template, 30 pmole of single pair primer, 2.5 mM of $MgCl_2$, 0.25 mM of dNTPs, and 1 U of *Taq* polymerase (Neurotics, Deajeon, Korea). The reaction conditions for PCR consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of amplification at 95°C for 30 s, annealing at either of 55°C, 50°C or 45ºC, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. PCR was conducted by using Tprofessional thermocycler (Biometra, Gottingen, Germany).

The first PCR for all primer sets was performed at 55°C. The optimal annealing temperature was then determined based on the banding pattern of the amplified product. Specifically, if no products were amplified or the product appeared faint, the annealing temperature was decreased from 55°C to 50°C or 45°C, and then no further tests of primers showing no products were conducted at annealing temperature of 45°C. The PCR amplification products were analyzed by electrophoresis on 2% agarose gels. The amplified DNA bands were stained with ethidium bromide solution and visualized on UV-transilluminator. PCR amplifications were conducted at least more than twice. Reproducible bands were scored visually.

Cloning of polymorphic fragments and designing of SCAR primers

For the conversion of ISSR polymorphisms to SCAR marker, polymorphic bands among the Korean ginseng cultivars were excised and purified from the agarose gel using QIAquick gel extraction kit (Qiagen). The collected fragments were ligated into T-blunt PCR cloning vector (SolGent, Daejeon, Korea). The recombinant plasmids were transformed into *Escherichia coli* strain (DH5α) by heat shock method. Clones of polymorphic ISSR markers were then sequenced bi-directionally by at Macrogen, Inc. (Seoul, Korea). The sequences were edited, after which the vector sequences were removed using Chromas ver. 1.42 (Griffith University, Brisbane, Australia). SCAR primer sets were designed using Primer3 (http://frodo.wi.mit.edu/primer3) based on sequence information from polymorphic ISSR marker sequences. The primer length was adjusted to 19 mer to 24 mer, and the optimum T_m value was adjusted to be 60°C with a range of 57°C to 63°C with minor modification.

RESULTS AND DISCUSSION

Selection of polymorphic ISSR markers

Initially, eighty-five ISSR primers were tested in order to detect polymorphisms among the six Korean ginseng cultivars and foreign ginseng. Of them, 34 ISSR primers generated interpretable polymorphic bands (Tables 2, 3

Table 2. List of inter simple sequence repeat primers showing genetic polymorphisms among six Korean ginseng cultivars, *Panax quinquefolius* and *P. notoginseng*

Primers	Sequence (5'→3')	Primers	Sequence (5'→3')
UBC-807	AGAGAGAGAGAGAGAGT	UBC-835	AGAGAGAGAGAGAGAGYC
UBC-808	AGAGAGAGAGAGAGAGC	UBC-836	AGAGAGAGAGAGAGAGYA
UBC-809	AGAGAGAGAGAGAGAGG	UBC-840	GAGAGAGAGAGAGAGAYT
UBC-810	GAGAGAGAGAGAGAGAT	UBC-842	GAGAGAGAGAGAGAGAYG
UBC-812	GAGAGAGAGAGAGAGAA	UBC-843	CTCTCTCTCTCTCTCTRA
UBC-813	CTCTCTCTCTCTCTCTT	UBC-845	CTCTCTCTCTCTCTCTRG
UBC-815	CTCTCTCTCTCTCTCTG	UBC-852	TCTCTCTCTCTCTCTCRA
UBC-817	CACACACACACACACAA	UBC-853	TCTCTCTCTCTCTCTCRT
UBC-818	CACACACACACACACAG	UBC-855	ACACACACACACACACYT
UBC-819	GTGTGTGTGTGTGTGTA	UBC-856	ACACACACACACACACYA
UBC-820	GTGTGTGTGTGTGTGTC	UBC-857	ACACACACACACACACYG
UBC-821	GTGTGTGTGTGTGTGTT	UBC-866	CTCCTCCTC CTCCTCCTC
UBC-822	TCTCTCTCTCTCTCTCA	UBC-868	GAAGAAGAAGAAGAAGAA
UBC-823	TCTCTCTCTCTCTCTCC	UBC-872	GATAGATAGATAGATA
UBC-824	TCTCTCTCTCTCTCTCG	UBC-878	GGATGGATGGATGGAT
UBC-826	ACACACACACACACACC	UBC-880	GGAGAGGAGAGGAGA
UBC-834	AGAGAGAGAGAGAGAGYT	UBC-891	HVHTGTGTGTGTGTGTG

Table 3. Characteristics of polymorphic inter simple sequence repeat primers among six Korean ginseng cultivars, *Panax quinquefolius* and *P. notoginseng*

Primers	SR (bp)	TL	NP	NB	PL[1)]	AT (°C)	Primers	SR (bp)	TL	NP	NB	PL[1)]	AT (°C)
UBC-807	180-1950	18	16	3	S	55	UBC-835	400-900	4	4	3	S	55
UBC-808	150-1000	9	8	3	S	55	UBC-836	80-1800	11	11	3	S	55
UBC-809	200-1200	9	8	3	S	55	UBC-840	220-1500	11	11	3	S	55
UBC-810	200-350	3	1	2	S	55	UBC-842	400-1600	13	13	3	S	55
UBC-812	300-700	7	4	3	S	55	UBC-843	420-1500	7	6	3	S	50
UBC-813	700-2400	5	4	3	S	55	UBC-845	1000-1500	2	1	2	S	50
UBC-815	700-1300	3	3	3	S	55	UBC-852	1000-1600	3	3	3	S	50
UBC-817	380-1200	6	5	3	S	55	UBC-853	450-1600	5	5	3	S	50
UBC-818	430-1400	5	3	3	S	55	UBC-855	300-1400	9	9	3	S	55
UBC-819	450-1200	2	1	2	S	55	UBC-856	350-1000	3	2	2	S	55
UBC-820	1000-1700	4	2	2	S	55	UBC-857	400-1200	6	4	3	S	55
UBC-821	630-1100	4	4	4	S, C	55	UBC-866	380-1600	11	5	3	S	55
UBC-822	630-3000	7	6	3	S	55	UBC-868	350-1500	16	16	4	S, C	55
UBC-823	680-1900	5	4	3	S	55	UBC-872	300-850	2	1	2	S	45
UBC-824	850-1600	3	3	2	S	55	UBC-878	600-1400	6	6	4	S, C	55
UBC-826	220-1200	8	6	3	S	55	UBC-880	250-1200	9	5	3	S	55
UBC-834	260-1800	12	11	3	S	55	UBC-891	410-1400	8	6	3	S	55

SR, size range of amplified fragments per assay unit; TL, total number of loci detected per assay unit; NP, number of polymorphic bands per assay unit; NB, number of banding patterns; PL, polymorphisms level; AT, annealing temperature.

[1)]'S' and 'C' indicate that polymorphisms occurred in 'species' level and in 'cultivar' level among six Korean ginseng cultivars, *P. quinquefolius*, and *P. notoginseng,* respectively.

and Fig. 1). The other primers generated monomorphic banding patterns, indistinguishable smear bands, or no bands.

With 34 ISSR primers, 197 polymorphic bands with average 5.8 polymorphic bands and 2.9 banding patterns per assay unit were generated in across six Korean ginseng cultivars and foreign ginsengs. 236 ISSR loci ranging from 80 bp (UBC-836) to 3,000 bp (UBC-822) with average 6.9 loci per assay unit were amplified (Table 3).

Two foreign ginsengs, *P. quinquefolius and P. notoginseng,* could be readily differentiated with 34 individual ISSR primers. However, monomorphic genotypes were generated with most of the primers among six Korean ginseng cultivars (Fig. 1). These results indicated that most polymorphic loci and banding patterns were derived from species level of *P. ginseng, P. quinquefolius,* and *P. notoginseng*. With only three ISSR primers, UBC-821, UBC-868, and UBC-878, polymorphic bands were generated among the six Korean ginseng cultivars, which could be also distinguished from foreign ginsengs (Fig. 2).

ISSR is popular marker systems, owing to their ability to detect polymorphisms without requiring the sequence information necessary for primer design. Because the appropriate sequence data or restriction fragment length polymorphism probes for primer design are not generally available for ginseng, ISSR has recently been conducted to characterize the genetic resources of germplasms, including Korean ginseng cultivars and other *Panax* species [21]. In this study, ISSR primers were tested to confirm the discrimination availability for Korean ginseng cultivars, via the application of a significant number of primers. Three species of *P. ginseng* containing the Korean ginseng cultivars*, P. quinquefolius,* and *P. notoginseng,* could be readily discriminated using most tested primers. This indicated that the discrimination of *Panax* species is no longer a particularly thorny problem, although Wen and Zimmer [1] previously reported on the phylogeny of *Panax* species, revealing that *P. quinquefolius* was very closely related to *P. ginseng*. However, polymorphisms among the Korean ginseng cultivars were detected using just 3 primers, even though a significant number of primers were actually brought to bear on the problem. This indicates that Korean ginseng cultivars have extremely narrow genetic diversity, as was previously noted by In *et al.* [21].

Because ISSR frequently yield sensitive results depending on the laboratory equipment, *Taq* polymerase

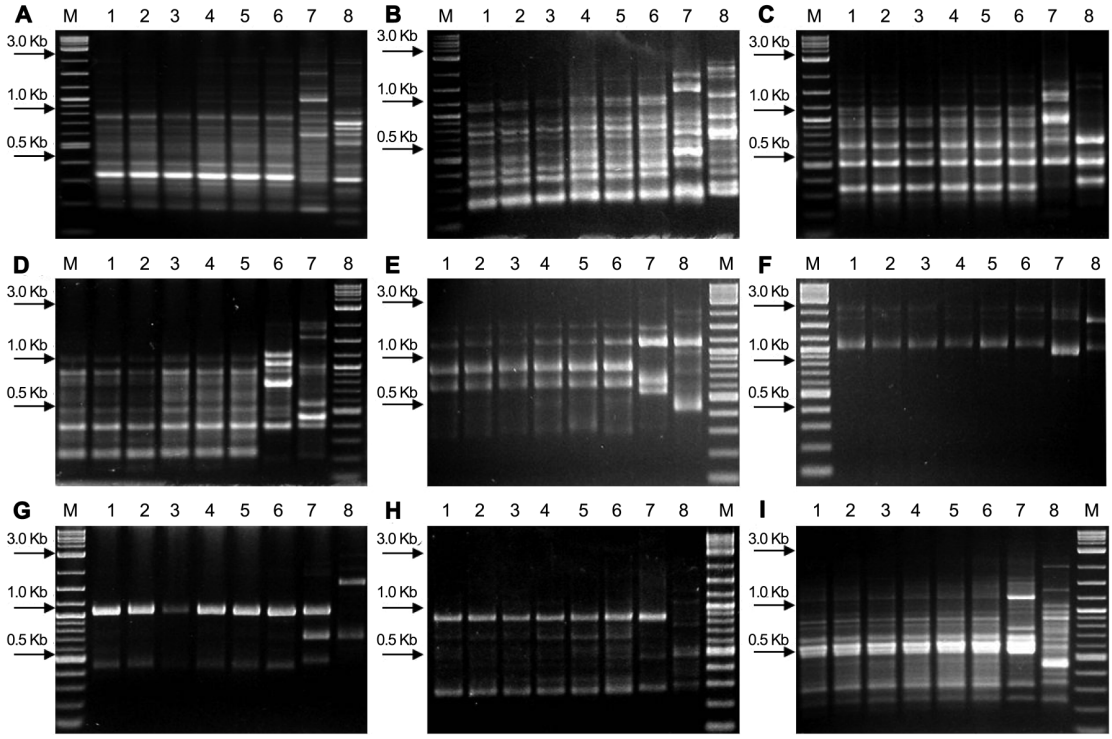

Fig. 1. Polymorphisms by the ISSR primers beteewn Korean ginseng cultivars and foreign ginseng. Lane 1, Chunpoong; lane 2, Yunpoong; lane 3, Gopoong; lane 4, Kumpoong; lane 5, Sunpoong; lane 6, Sunwon; lane 7, *Panax quinquefolius*; lane 8, *P. notoginseng*; lane M, DNA step ladder (2-Log DNA ladder; NEB, Beverly, MA, USA). (A) UBC-808, (B) UBC-809, (C) UBC-834, (D) UBC-840, (E) UBC-843, (F) UBC-852, (G) UBC-853, (H) UBC-872, and (I) UBC-880.

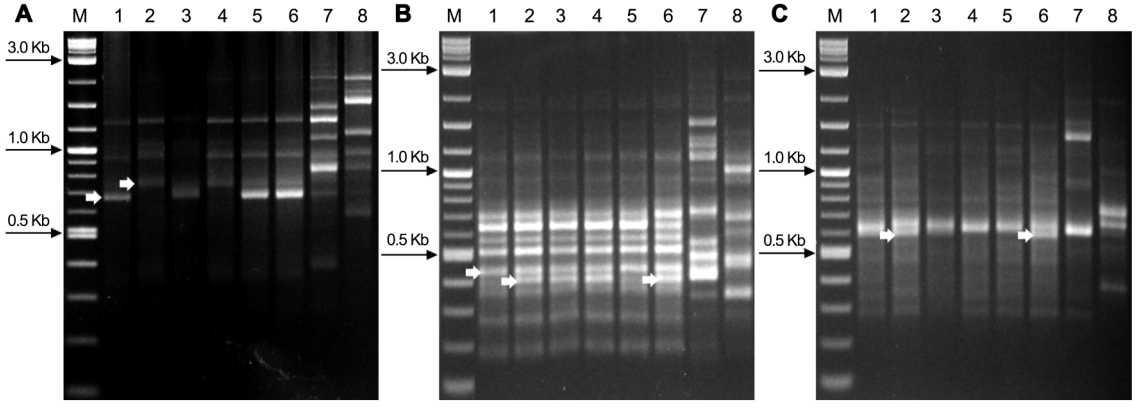

Fig. 2. Polymorphic banding patterns among Korean ginseng cultivars. Lane 1, Chunpoong; lane 2, Yunpoong; lane 3, Gopoong; lane 4, Kumpoong; lane 5, Sunpoong; lane 6, Sunwon; lane 7, *Panax quinquefolius*; lane 8, *P. notoginseng* ; lane M, DNA step ladder (2-Log DNA ladder; NEB, Beverly, MA, USA). (A) UBC-821, (B) UBC-868, and (C) UBC-878. These primers also discriminate Korean ginseng cultivars from two foreign ginsengs.

type, and so on, and because repeated results of the polymorphism may be detected inconsistently within the same cultivar, this technique needs to confirm the reproducibility of polymorphisms. Therefore, reproducibility tests were conducted to confirm the polymorphisms detected with the primers selected herein. However, this was done simply by increasing the number of individuals of each Korean ginseng cultivar without modifying any of the PCR conditions, as it is impossible to consider all of the experimental factors that might affect the results of the PCR amplifications. The results of the reproducibility tests demonstrated that the ISSR primers, which amplified polymorphic bands in an application of one individual for each Korean ginseng cultivar, ampli-

fied the expected polymorphic bands with reasonable reproducibility in three individuals of each Korean cultivar (data not shown).

Conversion of polymorphic ISSR markers to SCAR markers

Although relatively reproducible polymorphisms were identified from the ISSR results, the SCAR (sequence characterized amplified region) marker system was introduced in order to increase the reproducibility of polymorphisms. Polymorphic DNA fragments produced by the ISSR primers were cloned and sequenced for conversion to SCAR markers. Based on the sequences of the cloned ISSR fragments, several SCAR primer sets were originally designed and tested. Among them, one primer set (PgI821C650) converted from the about 650 bp polymorphic fragment detected with UBC-821 from Chunoong (Fig. 2A), successfully generated the anticipated polymorphism (Fig. 3A). The polymorphic bands were dominantly amplified in Chunpoong, Gopoong, Sunpoong, Sunwon, and *P. notoginseng*. The actual length of the polymorphic fragments from Chunpoong and the expected PCR size of the PgI821C650 were 655 bp and 647 bp, respectively (Table 4). The primer binding sites of PgI821C650 was as shown in Fig. 4.

Although, the PgI821C650 were successfully converted from the ISSR marker, it could not specifically discriminate ginseng cultivars. Thus, we applied six restriction enzymes (*Alu* I, *Hae* III, *Hinf* I, *Rsa* I, *Taq* I, and *Tsp509* I), so-called 'frequent cutters' having 4 bp as their recognition sequences, to the PCR products of PgI821C650 for detecting latent polymorphisms which might exist in the PCR products. As a result, the Sunwon cultivar was co-dominantly distinguished from other cultivars using *Taq* I restriction enzyme (Fig 3B). The PCR products of the Chunpoong, Gopoong, and Sunpoong cultivars were digested into two fragments accounting for approximately 400 bp and 250 bp, whereas the PCR product of the Sunwon cultivar was not digested. The *Taq* I restriction enzyme sites of PgI821C650 was as shown in Fig. 4.

One of the difficulties of conversion of randomly amplified polymorphism to SCAR marker is that desirable cloning of the polymorphic bands cannot be frequently conducted due to the heterogeneous nature of polymorphic bands. In the procedure of converting randomly amplified polymorphisms to SCAR markers, non-targeted sequences can frequently be generated from heterogeneously amplified fragments of similar size with the specific fragment, which might be contained in the polymorphic product determined as one band on the gel images. In addition, although the targeted sequence can be obtained, SCAR marker designed from the sequence does not frequently amplify the expected polymorphisms. In most cases, SCAR markers amplify polymorphic products of dominant types. This is because the ISSR techniques generally amplify dominant alleles, as their polymorphisms are caused by mismatches in primer

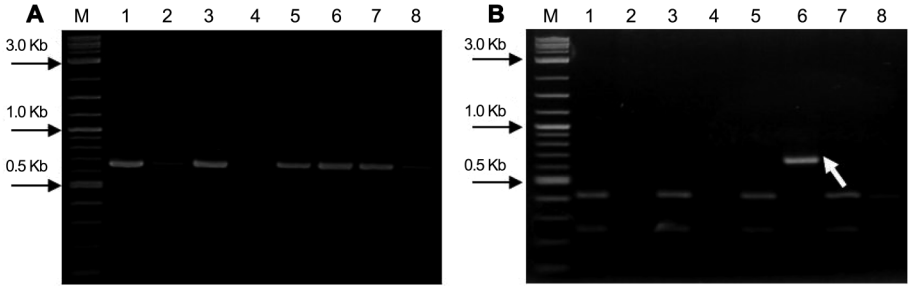

Fig. 3. The expected polymorphism amplified by the PgI821C650 among six Korean ginseng cultivars, *Panax quinquefolius*, and *P. notoginseng* (A) and Sunwon-specific polymorphism detected by treating *Taq* I restriction enzyme (B). Lane 1, Chunpoong; lane 2, Yunpoong; lane 3, Gopoong; lane 4, Kumpoong; lane 5, Sunpoong; lane 6, Sunwon; lane 7, *P. quinquefolius*; lane 8, *P. notoginseng*; lane M, DNA step ladder (2-Log DNA ladder; NEB, Beverly, MA, USA). An arrow indicates polymorphic fragments specific to Sunwon.

Table 4. Sequence characterized amplified region (SCAR) markers converted from polymorphic fragments of inter simple sequence repeat (ISSR) analysis

Information of cloned fragments			SCAR marker	Primer sequences (5'→3')	Expected size (bp)	Annealing temperature (°C)
Primer[1)]	Cultivar	Product size[2)]				
UBC-821	Chunpoong	655	PgI821C650	Forward: TGTGTGTGTGTTGAGAAAGC Reverse: TGTGTGTGTGTGTTCAAGC	647	65

[1)] Polymorphic ISSR marker.
[2)] Sequenced size (bp) of polymorphic fragments.

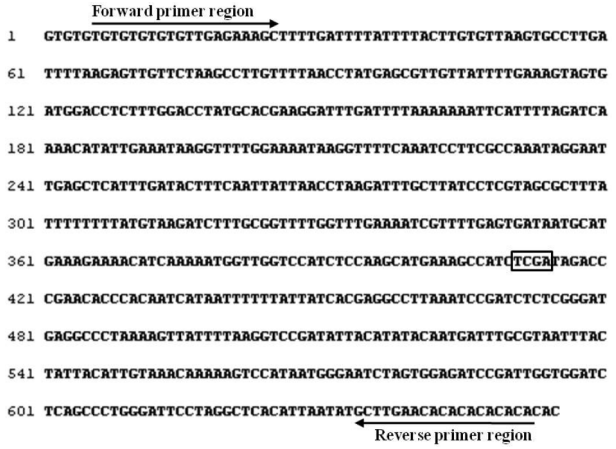

Fig. 4. Nucleotide sequence of the about 650 bp polymorphic fragment amplified by UBC-821 in Chunpoong (Fig. 2A) for sequence characterized amplified region marker, PgI821C650. Primer binding regions and directions are indicated with arrows. The solid box indicates the *Taq* I restriction site.

binding sites [18-20]. As the SCAR markers are generally designed in the internal region from the originally mismatched binding sites of random primers, if no variations exist in the internal region, the expected dominant polymorphisms frequently appear in the form of uniband between individuals. Likewise, in this study, many non-targeted sequences were generated from the cloned fragments, and most designed SCAR markers showed the unexpected PCR results. Thus, one SCAR marker was only converted from the polymorphic bands by those reasons. Nevertheless, we successfully converted the randomly amplified polymorphism to the SCAR marker, PgI821C650, showing the expected polymorphism, and developed the specific marker for Sunwon by treating restriction enzyme. The SCAR markers represent the first reported results of the conversion of random priming technique to SCAR markers in Korean ginseng cultivars. It will serve as useful DNA markers for identification of Korean ginseng cultivars, especially Sunwon, seed management, and molecular breeding program supplemented with marker-assisted selection.

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