Development of a single-nucleotide-polymorphism marker for specific authentication of Korean ginseng (*Panax ginseng* Meyer) new cultivar “G-1”

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1. Introduction

*Panax ginseng* Meyer is a deciduous perennial herb plant that belongs to the family Araliaceae. They are native to East Asia, while two species of them are found in North America. Ginseng has been used as a medicinal plant for over 2,000 years in Korea, China, and Japan as an immunostimulant, and acts as an agent to foster resistance to fatigue and stress [1–3]. The usage of ginsenoside-based medicinal products is increasing worldwide. Among the different species of ginseng, *P. ginseng* and *Panax quinquefolius* are the most popular for consumption, as well as for medicinal purposes.

Most of the commercial cultivation of *P. ginseng* has been centralized to South Korea and the northeastern part of China and Japan, whereas *P. quinquefolius* has been cultivated in China, Canada, and the United States. In South Korea, the ideal climatic conditions to grow ginseng plants in all four seasons favor the cultivation of many species of *Panax* for commercial purposes, such as...
as *P. ginseng*, *Panax notoginseng* (Chinese ginseng), *Panax japonicus* (Japanese ginseng), and *P. quinquefolius* L. [4]. Recently, there are a total of nine cultivars namely, Yunpoong, Gopoong, Sunpoong, Gumpoong, Chunpoong, Sunun, Sunone, Sunhyang, and Chungsun with features unique to Korean ginseng, which have been selected from three basic (varieties) lines (Jakyung, Chungkyung, and Hwangsook) using the pure-line-selection method [5]. A similar method was followed to develop the K-1, a new cultivar G-1 and registered with the Korea Seed & Variety Service (http://www.seed.go.kr).

Each Korean ginseng cultivar has unique features in relation to the improved agronomical properties, such as root yield, root shape, and disease resistance. Yunpoong has the highest root yield [6] and Chunpoong, Gopoong, Gumpoong have good root shapes. While considering the quality of red ginseng (steamed ginseng roots) in these cultivars, Chunpoong, Gumpoong has the highest root yield of Chun Sam and it is considered the first-grade ginseng followed by Gumpoong, Gopoong, Yunpoong, and Sunpoong. Pertaining to the ginsenoside unit content and total content of ginsenoside in 6-year-old ginseng roots, the ginsenoside content is higher in the order of Gopoong, Yunpoong, Chunpoong, Gumpoong, and Sunpoong [7]. The Korean ginseng cultivar G-1 was developed in 2012 and the improved agronomical properties, such as root yield, root shape, and disease resistance were also analyzed (data not shown).

These cultivars are grown in mixed ginseng fields, and are also sold mixed with other *Panax* species in the market. Therefore, the development of a valid authentication method is necessary for the preservation of these varieties, and to protect the rights of farmers and consumers. Although the medicinal components and efficacy of *P. ginseng* have been widely explored [8–11], there is little information available on the genome of *P. ginseng*, making the molecular identification of different cultivars difficult. However, with the development of robust molecular markers, such as polymerase chain reaction (PCR)—restriction fragment length polymorphism [12], single-strand conformation polymorphism [13], randomly amplified polymorphic DNA [14], sequence-characterized amplified region [15], intersimple sequence repeat-derived sequence-characterized amplified region [16], amplification-refractory mutation system (ARMS) [17], amplified fragment length polymorphism, and directed amplification of minisatellite region DNA [18] for the Korean ginseng cultivars, this difficulty is prevailed.

Traditional methods based on phenotypic observations have been used to identify the G-1 cultivar from the rest of the Korean ginseng cultivars, but morphological characteristics are often affected by environmental and developmental factors. Due to very similar phenotypical characteristics of these cultivars, the identification and authentication of G-1 becomes difficult especially during the seed-development and seedling stages. Thus, it is advantageous to use molecular methods to differentiate the ginseng cultivars. In this study, we investigated the possibility of using a single-nucleotide polymorphism (SNP) in 45S ribosomal DNA (rDNA) to differentiate ginseng cultivars. The nucleolar organizing regions (NORs) are cytologically observed as a secondary constriction containing many tandem repeats of 45S ribosomal ribonucleic acid genes [19]. The 45S rDNA sites were observed to be restricted to the NORs, although in some species, smaller or less active sites have also been detected outside the NORs [20]. Based on the SNP sites found for G-1, other Korean ginseng cultivars, and American ginseng, specific primers were designed and multiplex ARMS–PCR was conducted to authenticate these plants. This method based on DNA analysis is widely accepted as a means of identifying medicinal plants, because it is not affected by the growth stage and environmental conditions.

### Materials and methods

#### 2.1. Plant materials

Eleven ginseng samples (Table 2) were provided by the Ginseng Resource Bank. All voucher specimens were morphologically identified by Professor Woo-Saeng Kwon (Department of Oriental Medicinal Biotechnology, College of Life Sciences, Kyung Hee University).

| Ginseng sample | Voucher | Location | GenBank accession number of 45S |
|---------------|---------|----------|-------------------------------|
| Chunpoong     | GB001   | Kochang, Korea | KF727964 |
| Yunpoong      | GB002   | Kochang, Korea | KF727965 |
| Gopoong       | GB003   | Kochang, Korea | KF727966 |
| Sunpoong      | GB004   | Kochang, Korea | KF727967 |
| Gumpoong      | GB005   | Kochang, Korea | KF727968 |
| Sunun         | GB048   | Daejeon, Korea | KF727969 |
| Sunone        | GB073   | Daejeon, Korea | KF727970 |
| Sunhyang      | GB058   | Daejeon, Korea | KF727971 |
| K-1           | GB201   | Kochang, Korea | KF727973 |
| G-1           | GB101   | Kochang, Korea | KF727974 |
| G-1           | GB102   | Kochang, Korea | KF727975 |
| Panax quinquefolius | GB099 | USA | KF727975 |
| G-1           | GB101   | USA | KF727975 |
| P. quinquefolius | GB100 | USA | KF727975 |

#### Table 2

Ginseng plant samples used in this study

| Line | Cultivars | Color of stem                          | Color of berry | Leaf type                  | Registered date |
|------|-----------|----------------------------------------|----------------|----------------------------|-----------------|
| Jakyung | Yunpoong | Light violet                          | Red            | Having stipule             | 1998            |
|       | Gopoong   | Violet                                 | Red            | Long oval                  | 2000            |
|       | Sunpoong  | Violet                                 | Red            | Long oval                  | 2000            |
|       | Sunun     | Violet                                 | Red            | Long oval                  | 2004            |
|       | Sunone    | Violet                                 | Red            | Long oval                  | 2004            |
|       | Sunhyang  | Violet                                 | Red            | Long oval, occurrence of stipule | 2007        |
|       | K-1       | Violet                                 | Red            | Long oval, tippe            | 2012            |
|       | G-1       | Violet                                 | Red            | Occurrence of stipule       | 2013            |
| Chungkyung | Chunpoong | Green and violet spot in green        | Orange yellow  | Narrow elliptical          | 1998            |
|         | Chungsun  | Green                                  | Red            | Long oval                  | 2005            |
| Hwangsook | Gumpoong  | Green                                  | Yellow         | Long oval                  | 2000            |
2.2. DNA extraction and PCR amplification of ribosomal 45S region

The collected leaf samples were frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was isolated and purified using G Spin Kit for plants (iNtRON, Seongnam, Korea), as per the manufacturer’s instructions. The primer pairs used for amplification of the 45S region were 45SF (5′-GCG AGA ATT CCA CTG AAC CT-3′) and 45SR (5′-ACG AAT TCC CTC CGC TTA TTG ATA TGC TTA-3′).

PCR amplification was performed in a total volume of 20 μL, and the reaction mixture consisted of each of the primers at a concentration of 0.5 μM, 20 ng of template DNA, and 10 μL of 2× PCR premix (Genotech, Daejeon, Korea). The amplification profile consisted of one pre-denaturation cycle of 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, 2 min at 72°C, and a final extension at 72°C for 7 min. The PCR products were separated by 1.0% agarose gel electrophoresis and detected by ethidium-bromide staining under UV.

2.3. Sequencing and DNA sequence analysis

The PCR products were purified by PCQuick-spin (iNtRON) according to the manufacturer’s instructions, and the purified products were sequenced by Genotech. The DNA sequences of the ribosomal 45S region obtained in the sequencing experiments were compiled using SeqMan software (SeqMan 2, DNASTAR, Inc., 3801, Regent St, Madison, WI, 53705, USA) and edited using the BioEdit program (BioEdit 7.2.5, Ibis Biosciences, 2251 Faraday Avenue, Carlsbad, CA 92008, USA) [21]. Multiple sequence alignments were performed using the online ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/).

2.4. Design of specific primers

Three specific primers were designed based on the SNP site: KgF (a positive control specific to all cultivars of Korean ginseng), G-1F (specific to G-1 and Panax quinquefolius), and AgF (specific to P. quinquefolius) (Fig.1). We introduced an additional mismatch into

Table 3

| Primer name | Nucleotide sequence (5′→3′) | Position in 45S region |
|-------------|-----------------------------|------------------------|
| 45SF        | GCGAGAATCCACTGAACC          | [12]                   |
| 45SR        | AGGAATCCCTGCCATTGTAATGCTTA  | [12]                   |
| KgF         | GACCACCTTGGTGA (G→T)        | 170–186                |
| G-1F        | TCCTAAAACGAAAGCTCAGG (C→A) | 283–305                |
| AgF         | TCACTCTCTGGCAGGARCT         | 477–485                |

Note. Bold underlined nucleotide is the additional mismatch introduced via substitution of G for T and C for A.
Kgf and G-1F through the substitution of T for G and A for C at the third base from the 3' end. The reverse primer used in combination with these primers was 45SR. The sequences and orientations of common and specific primers are shown in Table 3 and Fig. 2, respectively.

2.5. ARMS-PCR

Based on the SNP site detected in the ribosomal 45S region, the primer pairs Kgf and 45SR were used as the universal primers for authentication of G-1 and P. ginseng. G-1F and AgF were designed for specific authentication of G-1 and P. quinquefolius, respectively. ARMS-PCR was conducted with all primer pairs for simultaneous molecular authentication of G-1, the other Korean ginseng cultivars, and P. quinquefolius. The 20-μL reaction mixture consisted of 20 ng of template DNA, 10 μL of 2 × PreMix DNA polymerase (Genotech), and the four primers (Kgf, G-1F, AgF, and 45SR) at concentrations of 0.13 μM, 0.13 μM, 0.13 μM, and 0.13 μM, respectively. The amplification profile consisted of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 62.5°C, 40 s at 72°C, and a final 7-min extension at 72°C.

3. Results and discussion

Molecular markers have become a popular tool for the identification and authentication of plant and animal species at the DNA level. Recently, a new class of biomarkers has become the method of choice for genomic identification and authentication. SNPs are the simplest form of sequence variation that can occur between different genomes. It essentially involves substitution of a nucleotide at one position to another one at a specific location [22,23], which is advantageous over previous methods of variation assessment. In plants, SNP research is still in its infancy, and SNPs have been rigorously documented only in a few species, including Panax species, and Acanthopanax species, and Acanthopanacis cor...
other ginseng cultivars by the simultaneous detection of the specific 45S rDNA region. We believe that this method will be a useful tool for the authentication of the new Korean ginseng cultivar G-1 and American ginseng.

Conflicts of interest

All authors declare no conflicts of interest.

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