Discrimination of Korean ginseng (Panax ginseng Meyer) cultivar Chunpoong and American ginseng (Panax quinquefolius) using the auxin repressed protein gene

Jong-Hak Kim2, Min-Kyeoung Kim1, Hongtao Wang2, Hee-Nyeong Lee2, Chi-Gyu Jin2, Woo-Saeng Kwon2, Deok-Chun Yang2,*

1 KM Fundamental Research Division, Korea Institute of Oriental Medicine, Daejeon, Korea
2 Department of Oriental Medicinal Material & Processing, College of Life Sciences, Kyung Hee University, Yongin, Korea

1. Introduction

Panax ginseng Meyer (Korean ginseng) of the Araliaceae family is a perennial herb, one of the most valuable medicinal plants used in Asia [1]. Most of the active ingredients found in ginseng are ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, phenolic compounds, and fatty acids [2]. The ginseng root is widely used as an antistress [3], antifatigue, anti-aging, anxiolytic [4], antioxidative [5], and antidiabetic [6] agent, and also for cardio-vascular protection and neuroprotection activities [7] in Asia. The Korean ginseng has a total of nine cultivars: “Yunpoong”, “Gopoong”, “Sunpoong”, “Gumpoong”, “Chunpoong”, “Sunun”, “Sunhyang”, and “Chungsun”, and these features are unique to Korean ginseng have been selected from three basic pure (varieties) lines (Jakyung, Chungkyung and Hwangsok) by selection method [8]. Among these nine cultivars, Chunpoong has excellent quality, high yield, high resistance to ‘rust rot disease’, and highest root yield of Chunsam (1st grade ginseng) when it is processed into red ginseng [9]. Therefore, we need to develop a method to distinguish Chunpoong from other species of ginseng roots. The most widely used Korean ginseng, American ginseng (P. quinquefolius L.), and their products have attracted to worldwide consumption and both have similar morphologies, making them hard to distinguish on sight [10]. Moreover, these cultivars are not only cultivated with mixed species in ginseng fields but are also sold mixed in the market. Therefore, the development of effective authentication methods is necessary for cultivar conservation as well as for protecting the rights of farmers and consumers. Although
the medicinal components and effects have been widely explored [11], there is little information on the genomics of *P. ginseng* and therefore molecular identification of different cultivars is difficult. Currently, the expressed sequence tag (EST) database is available and provides a marker gene in the plants, making it easy to locate insertions and deletions (InDel) [12,13]. Target gene expression and the gene nucleotide sequence of the auxin repressed protein gene was well known. At least in part, the auxin plant hormone (auxin) plays an important role in the growth and development of plants by controlling gene expression [14]. A number of initial or primary response genes induced by auxin have been identified and characterized [15].

An SNP for Chunpoong and American ginseng has been identified in the sequence data. Based on these SNP sites, the following specific primers were designed to distinguish Chunpoong and *P. quinquefolius* by multiplex polymerase chain reaction (PCR). The SNP was identified in Chunpoong and another eight cultivars, and then modified allele-specific primers were specifically designed to differentiate Chunpoong cultivar from *P. quinquefolius* via multiplex PCR. Therefore we propose an effective method for the genetic identification of the Chunpoong cultivar of *P. ginseng* and *P. quinquefolius* by multiplex PCR.

2. Materials and methods

2.1. Plant materials for sequencing data

Four-to-six-yr-old fresh leaves and roots of nine Korean ginseng samples such as “Yunpoong”, “Gopoong”, “Sunpoong”, “Gumpoong”, “Chunpoong”, “Sunun”, “Sunone”, “Sunhyang”, and “Chungsun”, and one American ginseng, (*P. quinquefolius*, violet-stem line, Jakyung)—a total of ten samples—were selected (Table 1). All voucher specimens were morphologically identified by a ginseng taxonomist and

| Ginseng Sample | Voucher | Location    | GenBank accession number of auxin repressed gene |
|----------------|---------|-------------|-----------------------------------------------|
| Chunpoong      | GB001   | Kochang, Korea | JQ396657                                     |
| Yunpoong       | GB002   | Kochang, Korea | JQ396653                                     |
| Gopoong        | GB003   | Kochang, Korea | JQ396654                                     |
| Sunpoong       | GB004   | Kochang, Korea | JQ396655                                     |
| Gumpoong       | GB005   | Kochang, Korea | JQ396656                                     |
| Sunun          | GB008   | Daejeon, Korea | JQ396652                                     |
| Chungsun       | GB007   | Daejeon, Korea | JQ396649                                     |
| Sunone         | GB004   | Daejeon, Korea | JQ396651                                     |
| Sunhyang       | GB007   | Daejeon, Korea | JQ396650                                     |
| *P. quinquefolius* | GB009 | USA                  | JQ396648                                     |

![Universal primer for ginseng](image1.png)

![Specific primer for American ginseng](image2.png)

![Specific primer for Chunpoong](image3.png)

Fig. 1. Comparison of the Auxin repressed protein gene sequence of nine cultivars of Panax ginseng and *P. quinquefolius*. The specific primers designed in auxin repressed protein gene. 1F and 2R are universal primers for ginseng. 3F is a positive primer specific to *P. quinquefolius*. 4F is a positive primer specific to Chunpoong.
were deposited in the Korean Ginseng Center and Ginseng Resource Bank, Kyung Hee University, Republic of Korea.

2.2. Selected EST-gene

We developed ginseng molecular markers for randomly selected EST sequences of high expression-, stress-, color-, and saponin-related genes using feature lists of ESTs (http://www.bioherbs.khu.ac.kr/).

2.3. DNA isolation and PCR amplification of auxin repressed protein gene

The collected leaf samples were frozen in liquid nitrogen and ground into fine powder. Genomic DNA was isolated and purified using a plant DNA extraction kit (GeneAll, General Bio System, Seoul, South Korea). The primer pairs used for amplification of auxin repressed protein gene were ARPF1 (5′-CTGGCAAGTTGAGGAAATGC) and ARPR1 (5′-CCAGAAACACCTGCTCGTGC) and ARPF2 (5′-CAAACAGCAACGCTACTCGCA) Universal primer and ARPR2 (5′-CTTGGCAAGTTCAGGAAGATG) Universal primer. The concentration of DNA was measured spectrophotometrically. Modification of genomic DNA for the multiplex PCR was performed using the allele-specific multiplex PCR (ARMs-PCR) method (Fig. 3).

2.4. Sequencing and DNA sequence analysis

PCR products were purified according to the manufacturer’s instructions using the PCR product purification kit (GeneAll, General Bio System). The purified products were sequenced by Genotech Inc. The DNA sequences of the auxin repressed protein gene obtained by sequencing were compiled using SeqMan software, and edited by the BioEdit program [16]. Multiple sequence alignments were performed using an online ClustalW2 program (EMBL-EBI, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, UK) (http://www.ebi.ac.uk/Tools/clustalw2/).

2.5. Design of specific primers

On the basis of detected DNA polymorphisms, specific primers were designed for the Chunpoong cultivar, *P. quinquefolius*, and other Korean ginseng, respectively (Fig. 1). Based on the specific SNP site, only one mismatch occurred in the 3′ terminus; a specific primer was designed for Chunpoong whereas primers 4F and 3F were designed for specific identification of *P. quinquefolius* based on a specific 5 bp-deletion. Primer 1F, another sense primer of 2R, was designed to provide a positive control for nine ginseng cultivars and *P. quinquefolius*. The sequences and orientations of specific and common primers are shown in Table 2 and Fig. 2, respectively.

2.6. Amplification refractory mutation system-PCR

The SNP was identified in Chunpoong ginseng, *P. quinquefolius*, and other varieties using the allele-specific multiplex PCR. Based on the detected SNP site in the auxin repressed protein gene, the primer pairs 1F and 2R were designed as universal primers for authentication of Chunpoong and other Korean ginseng cultivars and *P. quinquefolius*. Primers 3F and 4F were designed for specific authentication of *P. quinquefolius* and Chunpoong, respectively. Using four primer pairs, amplification refractory mutation system-PCR (ARMs-PCR) was carried out and varieties of ginseng and *P. quinquefolius* were subjected to molecular authentication. The sixteen samples were confirmed by using Multiplex PCR (Fig. 3).

The 20 μL reaction mixture consisted of 50 ng of template DNA and 10 μL of 2X PreMix DNA polymerase (Genotech). The concentration of all four primers, 1F, 2R, 3F, and 4R was 0.5 μM. The amplification profile consisted of 5 min at 94°C, 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 resolved on a 1.0% agarose gel and detected by ethidium bromide staining under UV.

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**Fig. 2.** A schematic diagram of the auxin repressed protein gene and the positions of the specific primers used for multiplex polymerase chain reaction. The primer pairs 1F and 2R were designed as universal primers for authentication of Chunpoong and other Korean ginseng cultivars and *P. quinquefolius*. 3F and 4F were designed for specific authentication of *P. quinquefolius* and Chunpoong.
simultaneously. The identity of all 16 samples was assessed using ARMS-PCR. P. quinquefolius authentication of Chunpoong, other Korean ginseng cultivars, and 3F and 4F were designed as universal primers for authentication of Chunpoong and other Korean ginseng cultivars and 2R were designed as universal primers for authentication of Chunpoong and other ginseng cultivars, by simultaneously detecting the specific amplification products of 489 bp and 215 bp, respectively (Fig. 4).

As expected, PCR in Chunpoong and P. quinquefolius samples using a combination of PCR primers 3F and 4F yielded the amplification products of 489 bp and 215 bp, respectively (Fig. 4). A large number of ginseng samples were tested by multiplex PCR, and the PCR was repeated several times for experimental verification of the reproducibility of the results. Thus, we can draw a conclusion that Chunpoong and P. quinquefolius can be clearly discriminated from the other ginseng cultivars, by simultaneously detecting the specific auxin repressed protein gene.

2.7. Test samples

The 27 samples of Korean ginseng and three samples of American ginseng, a total of 30 samples, are grown by KT&G Public Corporation, Republic of Korea.

3. Results

3.1. Morphological characteristics of nine Korean ginseng cultivars

Jakyung, Chungkyung and Hwangsook are three of the most common varieties of Korean ginseng. 1) Jakyung ("Yunpoong", "Gopoong", "Sunpoong", "Sunun", "Sunone" and "Sunhyang") have violet stems and red fruits. 2) Chungkyung ("Chunpoong", "Chunsun") have orange-yellow fruits, but Chunpoong is green with light violet. 3) Hwangsook ("Gumpoong") has green stems and yellow fruits (Table 3).

3.2. Alignment of DNA sequences of the auxin repressed protein gene

The auxin repressed protein gene of nine ginseng cultivars was amplified using primers and determined to be 1,107 bp. These results from sequence alignment showed that excluding Chunpoong the specific single nucleotide polymorphism (SNP) site and auxin repressed protein gene from different ginseng varieties are almost the same. Chunpoong contains adenine (A) at the 425th nucleotide position, but the other varieties contained guanine (G) at the same site. The GenBank accession numbers for the auxin repressed protein gene of nine Korean ginseng cultivars and American ginseng were JQ396657 (Chunpoong), JQ396653 (Yunpoong), JQ396654 (Gopoong), JQ396655 (Sunpoong), JQ396656 (Gumpoong), JQ396652 (Sunun), JQ396649 (Chungsun), JQ396651 (Sunone), JQ396650 (Sunhyang), and JQ396648 (P. quinquefolius), respectively.

3.3. Multiplex PCR

To validate the molecular markers which we developed based on this mutation and the Chunpoong-specific SNP site identified in auxin repressed protein gene, four primer pairs, 1F, 2R, 3F, and 4R were designed to authenticate Chunpoong, other Korean ginseng cultivars and P. quinquefolius, by multiplex PCR. It was predicted that the combination of the four primer pairs would generate fragments for different cultivars, Chunpoong, other Korean ginseng cultivars, and P. quinquefolius yielded the universal band of 609 bp amplified by primers 1F and 2R from the auxin repressed protein gene.

As expected, PCR in Chunpoong and P. quinquefolius samples using a combination of PCR primers 3F and 4F yielded the amplification products of 489 bp and 215 bp, respectively (Fig. 4).

3.4. Analysis of 30 different samples for the conformation

We tested 30 pure line samples from KT&G Public Corporation to confirm that the identified marker is working effectively. For the...
test samples, DNA was collected and the experiments performed as outlined in the Methods section. Samples 1, 2, 7, 14, 15, and 20 were confirmed as Chunpoong, which produces the amplicons of 200 bp and 600 bp. Samples 3, 4, 5, 10, and 16 were confirmed to be American ginseng by production of amplicons of 400 bp and 600 bp. A 600 bp amplicon was found, which confirmed Korean ginseng (except Chunpoong), in Samples 6, 8 and 9, 12, 13, 17, 18, and 19. Therefore, in the case of using the positive marker there is a 100% chance of authenticating Chunpoong (hit point Chunpoong available / Chunpoong sample score = 3/3 × 100 = 100). The error rate for the Chunpoong was 3.7% (Chunpoong error scores / outside Chunpoong sample score = 1/27 × 100 = 100; Fig. 3).

4. Discussion

Traditional authentication of ginseng, which has relied on morphological and histological differences, is limited and quite often unreliable. In addition, this method is not recommended since it requires large quantities of material that are significantly affected by environmental growth conditions as well as storage conditions [17].

In recent years, several technologies have been developed for authentication of ginseng, including DNA molecular markers. Benefiting from the advances in molecular biotechnologies in the past few decades, DNA molecular markers have become popular means for authentication of ginseng (e.g., RAPD, ISSR, RFLP, etc.).

In this study the focus was on the utilization of specific SNP markers using ESTs of ginseng cultivars based on the partial coding and noncoding sequences [18–20]. In order to develop molecular marker for Korean ginseng, auxin repressed protein gene was analyzed. In the Chunpoong cultivar, an SNP of Chunpoong was established by using a multiplex PCR assay. We believe that this method may serve as a useful tool and selection marker for ginseng authentication.

Fig. 4. Multiplex allele-specific polymerase chain reaction (PCR) products of ginseng materials. Lane M: 1 kb DNA ladder; Lane 1: Chunpoong; Lane 2: Korean ginseng; Lane 3: American ginseng. The Chunpoong, other Korean ginseng cultivars, and P. quinquefolius yielded the universal band of 600 bp amplified by primers 1F and 2R from the auxin repressed protein gene. The Chunpoong and P. quinquefolius, assessed by PCR using the combination of 3F and 4F primers, yielded 489 bp and 215 bp amplicons, respectively.

Conflicts of interest

All authors have no conflicts of interest.

Acknowledgments

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