J Ginseng Res 40 (2016) 395–399

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research article

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

Jong-Hak Kim [2,☆], Min-Kyeoung Kim [1,☆], Hongtao Wang [2], Hee-Nyeong Lee [2], Chi-Gyu Jin [2], Woo-Saeng Kwon [2], Deok-Chun Yang [2,*]

[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*

ARTICLE INFO

Article history:
Received 23 January 2015
Received in Revised form
3 August 2015
Accepted 3 December 2015
Available online 17 December 2015

Keywords:
multiplex PCR
Panax ginseng
P. quinquefolius
polymorphism

ABSTRACT

Background: Korean ginseng (*Panax ginseng*) is one of the most important medicinal plants in the Orient. Among nine cultivars of *P. ginseng*, Chunpoong commands a much greater market value and has been planted widely in Korea. Chunpoong has superior quality "Chunsam" (1st grade ginseng) when made into red ginseng.

Methods: A rapid and reliable method for discriminating the Chunpoong cultivar was developed by exploiting a single nucleotide polymorphism (SNP) in the *auxin repressed protein* gene of nine Korean ginseng cultivars using specific primers.

Results: An SNP was detected between Chunpoong and other cultivars, and modified allele-specific primers were designed from this SNP site to specifically identify the Chunpoong cultivar and *P. quinquefolius* via multiplex polymerase chain reaction (PCR).

Conclusion: These results suggest that great impact to prevent authentication of precise Chunpoong and other cultivars using the *auxin repressed protein* gene. We therefore present an effective method for the authentication of the Chunpoong cultivar of *P. ginseng* and *P. quinquefolius*.

Copyright © 2015, The Korean Society of Ginseng, Published by Elsevier. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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, antiamnestic [4], antioxidative [5], and antidiabetic [6] agent, and also for cardiovascular protection and neuroprotection activities [7] in Asia. The Korean ginseng has a total of nine cultivars: "Yunpoong", "Gopoong", "Sunpoong", "Gumpoong", "Chunpoong", "Sunun", "Sunone", "Sunhyang", and "Chungsun", and these features are unique to Korean ginseng have been selected from three basic pure (varieties) lines (Jakyung, Chungkyung and Hwangsook) 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

* Corresponding author. Department of Oriental Medicinal Material & Processing, College of Life Sciences, Kyung Hee University, 1732 Deogyeong-daero, Gigeung-gu, Yongin-si, Gyeonggi-do 17104, Korea.
E-mail addresses: dcyang@khu.ac.kr, deokchunyang@yahoo.co.kr (D.-C. Yang).
☆ Co-first authors. These authors contributed equally to this work.

p1226-8453 e2093-4947/$ – see front matter Copyright © 2015, The Korean Society of Ginseng, Published by Elsevier. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
http://dx.doi.org/10.1016/j.jgr.2015.12.002

Table 1
Ginseng samples used in this study

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	GBD048	Daejeon, Korea	JQ396652
Chungsun	GBD073	Daejeon, Korea	JQ396649
Sunone	GBD043	Daejeon, Korea	JQ396651
Sunhyang	GBD058	Daejeon, Korea	JQ396650
P. quinquefolius	GB099	USA	JQ396648

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

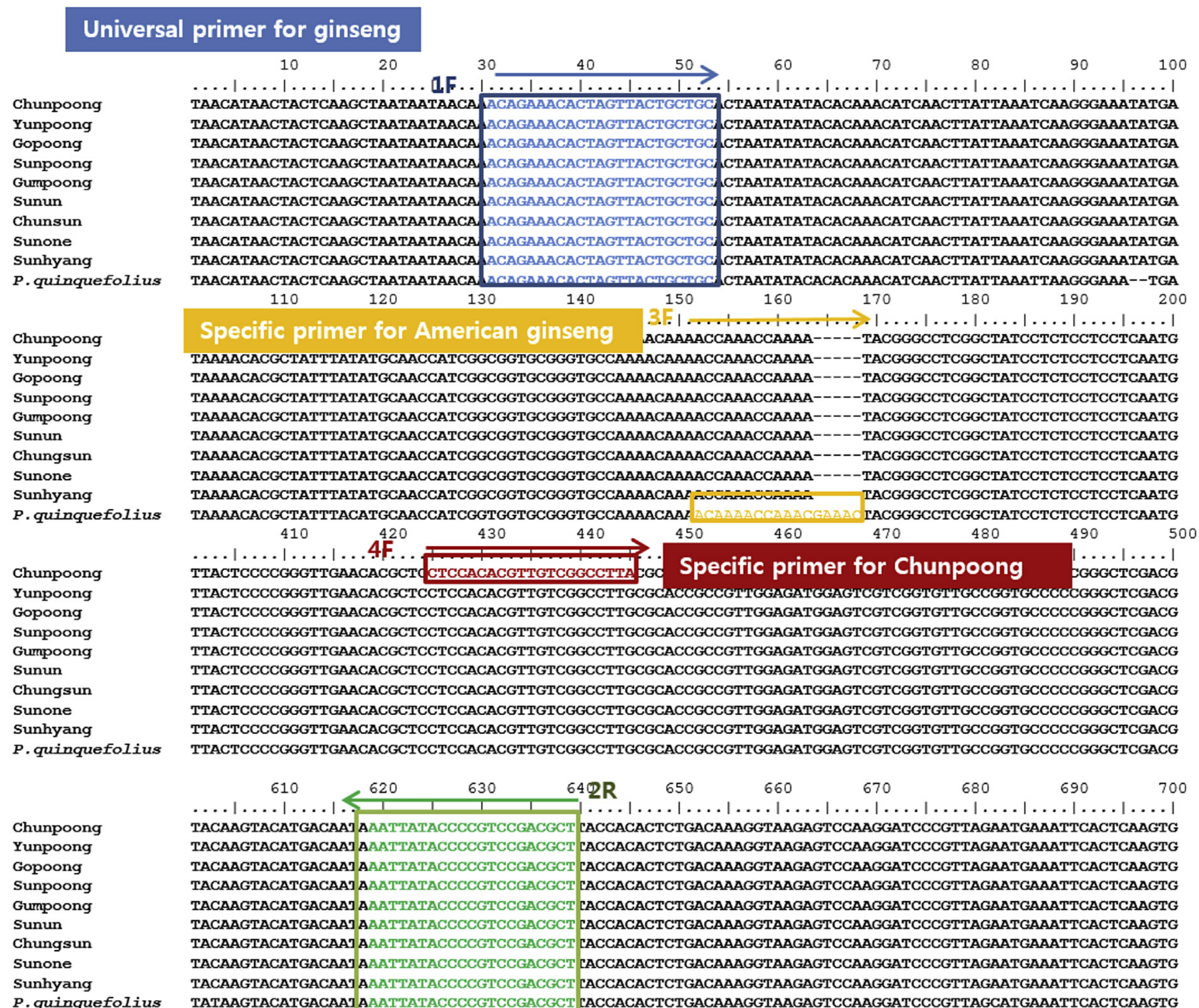

Fig. 1. Comparison of the *Auxin repressed protein* gene sequence of nine cultivars of *Panax ginseng* and *P. quinquefolius*. The specific primers designed in *auxin repressed protein* gene. 1F and 2R are universal primers for ginseng. 3F is a positive primer specific to *P. quinquefolius*. 4F is a positive primer specific to Chunpoong.

Table 2
Oligonucleotide sequences of primers used in this study

Primer name	Nucleotide sequence (5′–3′)	Position in *auxin repressed protein* gene
ARPF1	CTTGGCAAGTTCAGGAAGATG	Universal primer
ARPR1	CAAACAGCAACGCTACTCGCA	Universal primer
1F	ACAGAAACACTAGTTACTGCTGC	31–53
2R	AGCGTCGGACAAAATATAATT	639–619
4F	CTCCACACGTTGTCGGCCT**A**A (T-A)	425–445
3F	ACAAACAAAACCAAACGAAAC	31–53

Bold underlined nucleotide is the additional mismatch introduced via substitution of A for T

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 (Gene All, General Bio System, Seoul, South Korea). The primer pairs used for amplification of *auxin repressed protein* gene were ARPF1 (5′-CTT GGC AAG TTC AGG AAG ATG-3′) and ARPR1 (5′-CAA ACA GCA ACG CTA CTC GCA-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, 50 ng of template DNA, and 10 μl of 2X PCR premix (Genotech Inc., Daejeon, South Korea). The amplification profile consisted of 1 predenaturation 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 resolved on a 1.0% agarose gel and detected by ethidium bromide staining under UV.

The PCR products from the nine cultivars and *P. quinquefolius* were purified using a PCR DNA purification kit (Gene All, General Bio System, Seoul, South Korea) and then sequenced by Genotech, Inc (Daejeon, South Korea). The DNA sequences were registered in GenBank with the accession numbers JQ396648 and JQ396650–57 (Table 1).

2.4. Sequencing and DNA sequence analysis

PCR products were purified according to the manufacturer's instructions using the PCR product purification kit (GENEALL PCR SV, 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 55°C, 40 s at 72°C, and a final 7 min extension at 72°C.

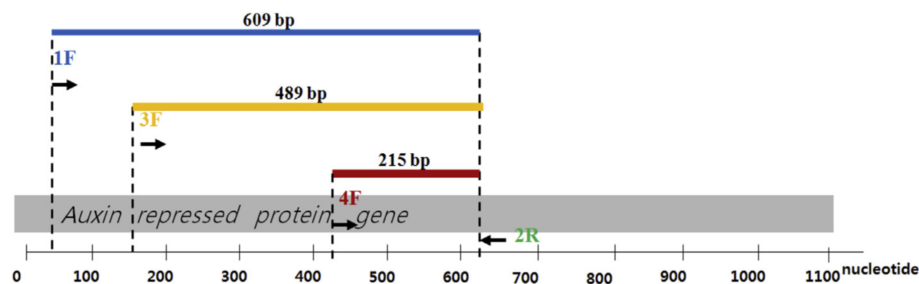

Fig. 2. A schematic diagram of the *auxin repressed protein* gene and the positions of the specific primers used for multiplex polymerase chain reaction. The primer pairs 1F and 2R were designed as universal primers for authentication of Chunpoong and other Korean ginseng cultivars and *P. quinquefolius*. 3F and 4F were designed for specific authentication of *P. quinquefolius* and Chunpoong.

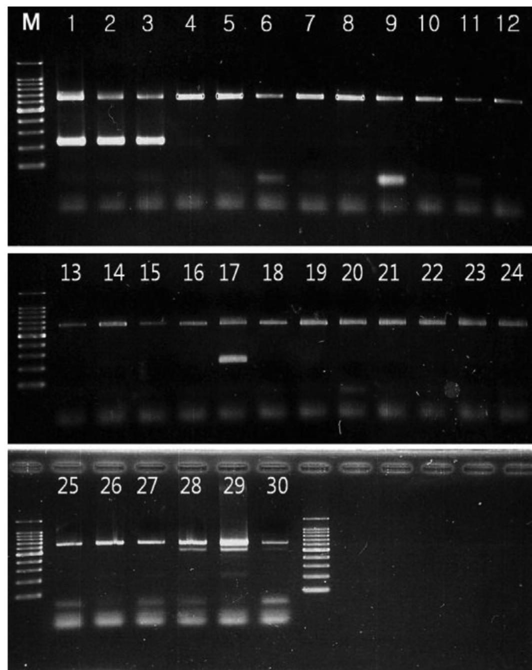

Fig. 3. Products of multiplex polymerase chain reaction (PCR) using primers 1F, 2R, 3F, and 4F and 30 different testing samples. Lane M: 1,000bp DNA ladder; Lanes 1–3: Chunpoong; Lanes 4–6: Yunpoong; Lanes 7–9: Gopoong; Lanes 10–12: Gumpoong; Lanes 13–15: Sunpoong; Lanes 16–18: Sunone; Lanes 19–21: Sunun; Lanes 22–24: Sunhyang; Lanes 25–27: Chungsun; Lanes 28–30: *P. quinquefolius*. 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. With the four primer pairs, amplification refractory mutation system-PCR (ARMS-PCR) was conducted for molecular authentication of Chunpoong, other Korean ginseng cultivars, and *P. quinquefolius* simultaneously. The identity of all 16 samples was assessed using ARMS-PCR.

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”, “Chungsun”) 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). 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.

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

Table 3
Main characteristics of aerial parts of 4-yr-old ginseng cultivars

Line	Cultivars	Color of stem	Color of berry	Leaf color at senescence	Leaf type	Ratio of multiple stem	Registered date
Jakyung	Yunpoong	Green	Red	Red	Having stipule	Above 50%	1998
	Gopoong	Violet	Red	Red	Long oval		2000
	Sunpoong	Violet	Red	Red	Long oval		2000
	Sunun	Violet	Red	Red	Long oval		2004
	Sunone	Violet	Red	Red	Long oval		2004
	Sunhyang	Violet in base	Red	Red	Long oval, occurrence of stipule		2007
Chungkyung	Chunpoong	Green and violet spot in base	Orange–yellow	Orange	Narrow elliptical		1998
	Chungsun	Green	Red	Red	Long oval		2005
Hwangsook	Gumpoong	Yellow	Yellow	Yellow	Long oval	7% or lower	2000

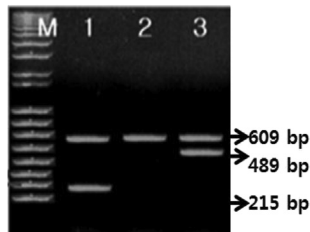

Fig. 4. Multiplex allele-specific polymerase chain reaction (PCR) products of ginseng materials. Lane M: 1 Kb DNA ladder; Lane 1: Chunpoong; Lane 2: Korean ginseng; Lane 3: American ginseng. The Chunpoong, other Korean ginseng cultivars, and *P. quinquefolius* yielded the universal band of 609 bp amplified by primers 1F and 2R from the *auxin repressed protein* gene. The Chunpoong and *P. quinquefoliusas*, assessed by PCR using the combination of 3F and 4F primers, yielded 489 bp and 215 bp amplicons, respectively.

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, 11, 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 $= 3/3 \times 100 = 100$). The error rate for the Chunpoong was 3.7% (Chunpoong error scores / outside Chunpoong sample score $= 1/27 \times 100 = 100$; 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 detected in the *auxin repressed protein* gene.

We performed simultaneous identification of the Chunpoong, other Korean ginseng cultivars, and *P. quinquefolius* using multiplex PCR. The method described in this study needs neither restriction nor the sequence analysis of PCR products, and the detection of the results only requires a simple gel-based assay, which is accessible in any molecular biology laboratory. Therefore, a simple and reliable method for simultaneous authentication of Chunpoong, other Korean ginseng cultivars, and *P. quinquefolius* 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.

Conflicts of interest

All authors have no conflicts of interest.

Acknowledgments

This work was supported by the YS personnel expenditure (grant number: 490009-1297) from the National Research Council of Science & Technology (NST) and the grant K15060 awarded to Korea Institute of Oriental Medicine (KIOM) from the Ministry of Education, Science and Technology (MEST), Korea. This research was supported by iPET (grant number: 11242-05-3-SB020), Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Republic of Korea. Thank you to KT&G Public Corporation, Republic of Korea for its support.

References

[1] Liu CX, Xiao PG. Recent advances in ginseng research in China. J Ethnopharmacol 1992;36:27–38.
[2] Lee FC. Facts about ginseng, the elixir of life. In: Elizabeth NJ, editor . Elizabeth, New Jersey, U.S.A.: Hollym International Corporation; 1992.
[3] Wang LC, Lee TF. Effect of ginseng saponins on cold tolerance in young and elderly rats. Planta Med 2000;66:144–7.
[4] Chen XC, Zhou YC, Chen Y, Zhu YG, Fang F, Chen LM. Ginsenoside Rg1 reduces MPTP-induced substantia nigra neuron loss by suppressing oxidative stress. Acta Pharmacol Sin 2005;26:56–62.
[5] Choi KT. Botanical characteristics, pharmacological effects and medicinal components of Korean *Panax ginseng* C.A. Meyer. Acta Pharmacol Sin 2008;29:1109–18.
[6] Xie JT, Mehendale SR, Li X, Quigg R, Wang X, Wang CZ, Wu JA, Aung HH, A Rue P, Bell GI, et al. Anti-diabetic effect of ginsenoside Re in ob/ob mice. Biochim Biophys Acta 2004;1740:319–25.
[7] Joo SS, Won TJ, Lee DI. Reciprocal activity of ginsenosides in the production of proinflammatory repertoire, and their potential roles in neuroprotection *in vivo*. Planta Med 2005;71:476–81.
[8] Kwon WS, Chung CM, Kim YT, Lee MG, Choi KT. Breeding process and characteristics of KG101, a superior line of *Panax ginseng* C.A. Meyer. J Ginseng Res 1998;22:11–7.
[9] Wang TS, Wang YP. New cultivars and rotation system of Korean ginseng. J Ginseng Res 2003;3:13–4.
[10] Chen CF, Chiou WF, Zhang JT. Comparison of the pharmacological effects of *Panax ginseng* and *Panax quinquefolium*. Acta Pharmacol Sin 2008;29:1103–8.
[11] Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharmacol 1999;58:1685–93.
[12] Sun H, Wang HT, Kwon WS, In JG, Lee BS, Yang DC. Development of molecular markers for new cultivar 'Chunpoong' in *Panax ginseng* C. A. Meyer associated with a Major Latex-like protein gene. Biol Pharm Bull 2010;33:183–7.
[13] Weiskirchen R, Siemeister G, Hartl M, Bister K. Sequence and expression of a glyceraldehyde-3-phosphate dehydrogenase-encoding gene from quail embryo fibroblasts. Gene 1993;128:269–72.
[14] Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin. Plant Cell 2007;9:1963–71.
[15] Abel S, Theologis A. Early genes and auxin action. Plant Physiol 1996;111:9–17.
[16] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT Nucleic. Acids Symp Ser 1999;41:95–8.
[17] Yip TT, Lau CN, But PP, Kong YC. Quantitative analysis of ginsenosides in fresh *Panax ginseng*. Am J Chinese Med 1985;13:77–88.
[18] Kim NH, Choi HI, Kim KH, Jang WJ, Yang TJ. Evidence of genome duplication revealed by sequence analysis of multi-loci expressed sequence tag-simple sequence repeat bands in *Panax ginseng* Meyer. J Ginseng Res 2014;38:130–5.
[19] Choi HI, Kim NH, Kim JH, Choi BS, Ahn IO, Lee JS, Yang TJ. Development of reproducible EST-derived SSR markers and assessment of genetic diversity in *Panax ginseng* cultivars and related species. J Ginseng Res 2011;35:399–412.
[20] Choi HI, Kim NH, Lee JK, Choi BS, Kim KD, Park JY, Lee SC, Yang TJ. Evolutionary relationship of *Panax ginseng* and *P. quinquefolius* inferred from sequencing and comparative analysis of expressed sequence tags. Genet Resour Crop Evol 2013;60:1377–87.