Development of Molecular Markers for the Determination of the New Cultivar ‘Chunpoong’ in *Panax ginseng* C. A. MEYER Associated with a Major Latex-Like Protein Gene

Hua Sun, a Hong Tao Wang, a Woo Saeng Kwon, a Jun Gyo In, b Bum Soo Lee, b and Deok Chun Yang a

a Department of Oriental Medicinal Material & Processing, College of Life Science, Kyung Hee University, 1 Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446–701, South Korea; and b Biopia, Co., Ltd., Yongin 449–598, South Korea.

Received August 4, 2009; accepted October 26, 2009; published online November 30, 2009

Chunpoong is one of the most valuable cultivars of *Panax ginseng* C. A. MEYER, and is widely grown in Korea and China. Insertion/deletion (InDel) markers and single nucleotide polymorphism (SNP) markers are useful tools for marker-assisted selections. The SNP marker for determinate Chunpoong was previously developed from the *nad7* gene of mtDNA by Wang et al. (2009) but was effective only on a limited range of cultivars. In this study, we studied the reasons for this limited application and developed new useful markers for application in Chunpoong-breeding programs. The new markers of InDel and SNP were designed in the major latex-like protein (MLP-like) gene which was highly expressed in 4-year-old Chunpoong expressed sequence tags (ESTs). To validate the marker in polymerase chain reaction (PCR), we used an InDel marker for identification of Chunpoong in the 70 *Panax* samples based on a double-blind test, and the success rate was 100%. For rapid and reliable assay of Chunpoong in numerous samples, we utilized an EvaGreen dye and melting curve method on real-time PCR. Furthermore, we designed an SNP marker that depended on the InDel region for more efficient detection of Chunpoong in real-time PCR. Compared with PCR-based assays, our Chunpoong SNP marker and real-time PCR assay offers a significant savings of time and labor in the analysis of large numbers of Chunpoong samples.

Key words *Panax ginseng*; Chunpoong; real-time polymerase chain reaction; major latex-like protein

Almost all *Panax* spp. (family Araliaceae) have been used as folk medicines in Korea and China. The most well-known species is *P. ginseng* C. A. MEYER, which was recorded in Chinese Materia Medica 2000 years ago. Korea has been dominant in the cultivation of *P. ginseng*, and nine new cultivars with good quality and high yield have been developed since 1972. Chunpoong is suitable to generate highly valuable “red ginseng” because it has good root shape. Thus, Chunpoong commands a much greater market value. However, the cultivar is frequently mixed-cultivated in farmhouses, and the seeds of Chunpoong are also sold mixed in the market. Therefore, it is essential to develop effective authentication methods to differentiate Chunpoong to protect the system of exchanging and circulating cultivars as well as the rights of consumers.

Molecular markers have demonstrated a potential to detect genetic diversity and to aid the management of plant genetic resources. In contrast to morphological traits, molecular markers can reveal differences among genotypes at the DNA level, providing a more direct, reliable and efficient tool for germplasm characterization, conservation and management. Polymerase chain reaction (PCR) and real-time PCR technologies have opened up increasing opportunities for detecting and studying genotypes in plants. For Korean ginseng cultivars, there have been few reports on molecular markers, including inter simple sequence repeat (ISSR), 1-5) PCR-restricted fragment length polymorphism (RFLP), 6-9) and amplification refractory mutation system (ARMS)-PCR. However, ISSR and PCR-RFLP have failed to develop a robust molecular marker for single-cultivar identification. In addition, Wang et al. (2009) developed Chunpoong single nucleotide polymorphism (SNP) marker in the *nad7* intron region of mitochondrial DNA, but the marker was effective only on a limited range of cultivars.

In recent years, numerous SNP-based markers have been developed from expressed sequence tags (ESTs), 9-12) and EST-PCR markers of plants have been reported. In this study, we developed a Chunpoong specific marker based on major latex-like protein (MLP-like) ESTs. Major latex-like proteins are present exclusively in latex and represent very good markers to investigate the regulation of the expression of laticifer-specific genes.

The aim of the present study was to develop Insertion/deletion (InDel)/SNP markers of Chunpoong in the downstream region of the MLP-like gene based on PCR, and to apply real-time PCR for raising the efficiency of the assay.

MATERIALS AND METHODS

**Plant Materials** All fresh ginseng leaves and dry roots were obtained from the Ginseng Genetic Resource Bank (Kyung Hee University, Korea). We used *P. ginseng* cultivars (‘Chunpoong,’ ‘Yunpoong,’ ‘Gopoong,’ ‘Gumpoong,’ ‘Sunpoong,’ ‘Sunwon,’ ‘Sunweon,’ ‘Sunhyang,’ ‘Chungsun,’ ‘Mimaki’) and several varieties (‘Hwangsook,’ ‘Ermaya,’ ‘Dumaya,’ ‘Biantiaoshen’), as well as *P. quinquefolius* and hybrid F1 (*P. ginseng* and *P. quinquefolius*).

**DNA Extraction and PCR Amplification** Total DNA was extracted from fresh leaves and roots using a Plant DNA isolation SV mini Kit (GeneAll, Korea). Specific primers were designed from MLP151 (EU939308.1) sequence. The PCR amplifications were performed in a Corbett PCR (Corbett Research, Sydney, Australia. Model: CG1-96). Each PCR was carried out in a total volume of 20 μl, containing...
20 ng of template DNA, 0.5 μM of each primer, 200 μM deoxyribonucleotide triphosphate (dNTPs), 1.5 mM MgCl₂, 1X PCR Buffer, and 1 U of DNA Polymerase (Enzymomics™, Korea). PCR amplifications were performed at 94 °C for 5 min, 35 cycles of 30 s at 94 °C, 30 s at 58 °C for annealing, and 30 s of extension at 72 °C, ending with 7 min at 72 °C.

**Real-Time PCR Amplification and Analysis** Real-time PCR was performed in a Rotor-Gene™ 6000 (Corbett Life Science, Australia). The reaction mixture consisted of 12 ng DNA, 5 μM of each primer, 200 μM of each dNTP, 1.5 mM MgCl₂, 2X mastermix (SensiMix HRM™, Quantace Ltd., Australia), and 0.4 μl EvaGreen, Tris ethylenediaminetetraacetic acid (TE) buffer in a total volume of 10 μl. The following PCR cycle profile was as follows: 10 min at 95 °C, followed by 40 cycles of a three-step thermal profile involving 15 s at 95 °C for denaturation and 20 s at 60 °C for combined annealing, as well as 10 s at 72 °C for extension (The Green channel setting ‘On’). The melt analysis conditions consisted of a ramp from 65 °C to 85 °C, rising by 0.1 °C at each step.

**DNA Walking** Adapter PCR was used to find the unknown sequences up- and downstream of MLP151 gene using a GenomeWalker Kit (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.). Genomic DNA was digested completely with two restriction enzymes, EcoRV and PvuII. The two enzymes produced blunt-end DNA fragments. Each batch of digested genomic DNA fragments was ligated with an adapter to create genomic libraries. Restriction enzyme-digested DNA fragments were ligated with adapters. The adapter-ligated genomic DNA fragments were used as templates to amplify the unknown DNA sequences. In the first cycle of PCR, DNA was amplified by the primer complementary to the DNA sequence. To increase the sensitivity, amplification product was used as nested PCR.

**Sequencing of the PCR Products** The PCR products were purified to remove primes and primer dimers using a PCR SV Kit (GeneAll, Korea). The amplified fragments of interest were excised from the 2% agarose gel and purified using an Agarose Gel DNA extraction kit (GeneAll, Korea). The fragments were then cloned into a pGEM-T Easy Vector System (Promega, U.S.A.) and transformed into *Escherichia coli* XL1-Blue cells. The plasmids were purified with a Plasmid SV mini kit (GeneAll, Korea). Sequencing was performed by Genotech Co., Ltd. (Genotech, Korea) (http://www.genotech.co.kr).

**Computer Analysis** Data on a total of 3706 ESTs (DC03, 4-year-old Chunpoong root ESTs) were obtained from the Ginseng Genetic Resource Bank (Kyung Hee University, Korea). An analysis chromatogram of clone sequences was produced using SeqManII, and the clustering sequences were generated using the Kalign 2.0 program (www.ebi.ac.uk/Tools/kalign/index.html). In addition, we used BioEdit 7.0 to confirm the local blast and assemble the ESTs to make contig using the CAP3 program. The PCR primers were designed using the Primer3 program (http://frodo.wi.mit.edu).

**RESULTS**

**Developing Marker for Detection Chunpoong** In the ESTs of Chunpoong 4-year-old root, the MLP-like gene was expressed highly and divided into two groups, MLP31 and MLP28 (data not shown). To obtain more information about the MLP-like gene sequences, we confirmed DNA walking in the MLP151 gene downstream region (described in Materials and Methods). We discovered that one primer pair can differentiate Chunpoong from Gopoong at the 326 bp fragment (Fig. 1C). The 326 bp fragment (named Chunpoong Positive Region-CPR, GQ979990) of Chunpoong was confirmed by cloning and sequencing. In the sequence, we found that the CPR has two regions (I and II) and 99% sequence similarity without InDels (Fig. 1B). To obtain a more specific fragment, we designed primers mlpmRF/TSP2R between the I and II regions, and conducted PCR (Table 1). Two fragments, 321 and 181 bp (GQ979992, GQ979991), were observed in Chunpoong, whereas two fragments, 321 and 682 bp (GQ979993, GQ979994), were observed in Gopoong (Fig. 1D). The results showed the primer set mlpmRF/TSP2R was effective at identifying Chunpoong.

**PCR Success Rate Comparison** To validate the marker, we conducted PCR among five cultivars and three Chinese cultivars (Table 2). The results showed that Chunpoong was distinguished from all other cultivars (Fig. 1E). In addition, we identified Chunpoong in the 70 *Panax* samples based on a double-blind test (Table 2, No. 1—12). The 181 bp fragment was amplified only in the Chunpoong genotypes. The 321 bp fragment was amplified in all samples except *P. quinquefolius* (Fig. 2). This result revealed 100% accuracy and reproducibility compared with the morphology and genotype in all ginseng samples. In addition, Chunpoong seeds and hybrid F1 were also correctly identified using the Chunpoong marker (Fig. 3, Table 2, No. 1 and 13).

**Application of Real-Time PCR** All experiments used twelve different samples (Table 2, No. 1—12). Figure 4A shows that Chunpoong had only one major peak and the other cultivars had two peaks in melting curves with Eva-Green dye. The Chunpoong melting Tm was 76.75 °C and the melting Tm of the other cultivars was 76.33 to 76.67 °C. When viewed by agarose gel electrophoresis, a single fragment of 181 bp was amplified in Chunpoong, and 321 bp fragments were strongly amplified in the other cultivars (Fig. 4B). Compared with PCR, *P. quinquefolius* was not amplified in real-time PCR because real-time PCR was limited to amplifying more than 400 bp fragments. In addition, only one major fragment was amplified in Chunpoong in real-time PCR because real-time PCR master mix reacted with a smaller major fragment first, and the DNA concentration was too low to amplify with both fragments. For efficient detection of Chunpoong in real-time PCR, we designed an SNP marker (181F/TSP2R) by CPR region. After amplification with those primers, one fragment of 174 bp was observed in Chunpoong, and other cultivars were not amplified (Fig. 5, Table 1). The melting curve result showed that Chunpoong was positive and almost all other samples were negative, but some samples started amplification from 42 cycles (Figs. 4C, D).

**DISCUSSION**

Our MLP-like protein belonged to the Bet v I family of proteins, and showed the highest homology with MLP31 and MLP28 in *A. thaliana* compared with the other MLP proteins. The function of the MLP proteins in plants is unknown, but a few patterns of expression show similarity with
Fig. 1. Chunpoong Positive Region in MLP-Like Gene

(A) The black, white and dash boxes indicate CPR (Chunpoong positive region), 321 bp and 181 bp regions, respectively. (B) Alignment of sequences showed high homology between regions I and II of CPR. The locations of the primers are indicated by a black box. (C) The 326 bp fragment was amplified by the TSP2 and mlpmR primers in Chunpoong. (D) Using mlpmRF and TSP2R primers resulted in a 181 bp that was a Chunpoong-positive fragment. The 321 bp fragment was common to both alleles and was used as internal control. (E) PCR result showed Chunpoong marker distinct from other cultivars of Korean ginseng and Chinese ginseng. M 1kb DNA ladder, 1: Chunpoong, 2: Gopoong.

Table 1. Primer Sequences Used for Identification of Chunpoong

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
</tr>
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<tr>
<td>181F</td>
<td>GGC TCG ATA TAT GTA CGT A</td>
</tr>
<tr>
<td>mlpmRF</td>
<td>ATC AAT GGG CTC GAT ATA TG</td>
</tr>
<tr>
<td>TSP2R</td>
<td>CCA CCT GCA CCA TAA GTG ACA A</td>
</tr>
</tbody>
</table>

Table 2. Ginseng Samples Used in This Study

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Classification</th>
<th>Tissue</th>
<th>Total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chunpoong</td>
<td>Korean cultivar</td>
<td>Leaf/Seed</td>
<td>11/17</td>
</tr>
<tr>
<td>2</td>
<td>Yunpoong</td>
<td>Korean cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Gopoong</td>
<td>Korean cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Gumpoong</td>
<td>Korean cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Sunpoong</td>
<td>Korean cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>Sunwon</td>
<td>Korean cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>Sunweon</td>
<td>Korean cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>Sunhyang</td>
<td>Korean cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Chungsun</td>
<td>Korean cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>Hwangsookjng</td>
<td>Korean variety</td>
<td>Leaf</td>
<td>9</td>
</tr>
<tr>
<td>11</td>
<td>Mimaki</td>
<td>Japanese cultivar</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>P. quinquefolius</td>
<td>American ginseng</td>
<td>Leaf</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>Hybrid F1</td>
<td>Korean and American ginseng</td>
<td>Leaf</td>
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<td>14</td>
<td>Ermaya</td>
<td>Chinese cultivar</td>
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<td>15</td>
<td>Damaya</td>
<td>Chinese cultivar</td>
<td>Dry root</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>Biantiaoshen</td>
<td>Chinese cultivar</td>
<td>Dry root</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 2. PCR Analysis of 70 Ginseng Samples Based on a Double-Blind Test

P. ginseng cultivars (Chunpoong: 2, 7, 9, 18, 20, 32, 36, 38, 45, 50, 55, Yunpoong: 12, 29, 35, 49, Gopoong: 6, 19, 33, 39, 58, Gumpoong: 13, 23, 46, 60, 70, Sunwon: 8, 11, 30, 47, 65, Sunweon: 1, 14, 24, 51, 68, Sunhyang: 25, 43, 57, 62, 69, Chungsun: 4, 16, 27, 44, 61, Mimaki: 10, 26, 42, 52, 59, P. ginseng varieties (Hwangsookjng: 5, 17, 22, 31, 37, 54, 56, 64, 67) and P. quinquefolius (15, 28, 34, 40, 48).
some of the intracellular pathogenesis-related (IPR) proteins, and the two proteins are indeed related.\(^{16}\) In a previous study, MLP-like gene was applied by EST-derived marker in *Rhododendron*.\(^{17}\)

In a special CPR region, InDel and SNP markers were designed for identification of Chunpoong. The effect of InDel marker was revealed to be the same as multiplex-PCR due to the CPR specific region (Figs. 1A, B). The major fragments of 181, 321, and 682 bp revealed marker specific bands of Chunpoong, *P. ginseng*, and *P. quinquefolius*, respectively. If using multiplex-PCR then at least four primers must be used, but our InDel marker uses only two primers. Using the characteristics of the InDel marker, we can identify hybrid F1, which is cross-cultivated *P. ginseng* and *P. quinquefolius*. To obtain a more effective identification of hybrid F1, Hot-taq polymerase can be used to confirm PCR. Therefore, InDel marker is special and useful for identification Chunpoong, *P. quinquefolius*, and hybrid F1.

Until now, the real-time PCR method has been successfully used for the detection of celery in food,\(^{18}\) *Phytophthora cryptogea* on *Gerbera jamesonii*,\(^{19}\) the age of wines,\(^{20}\) and GM papayas\(^{21}\) by melting curve analysis. Compared with normal PCR, real-time PCR assay is highly sensitive, specific, fast, and convenient since gel electrophoresis is not needed. The study presented here developed an SNP marker for easy identification of Chunpoong in real-time PCR using a melting curve method because all genotypes were amplified by InDel marker but only SNP marker was amplified in Chunpoong. Moreover, we used EvaGreen dye to do the melting curve assay because EvaGreen has wider linear ranges in quantitative analysis and better reproducibility in the separations of dsDNA fragments compared with SYBR Green I.\(^{22}\) In ginseng molecular marker research, this is the first report on the application of an allele-specific marker in real-time PCR for assaying numerous samples.

For assaying large numbers of samples, the DNA extraction method used is extremely important because time and
cost are the most important considerations. Many rapid DNA extraction methods have been developed and applied thus far in plant breeding programs. If a rapid DNA extraction method and real-time PCR are used to identify Chunpoong, the process would become faster and cheaper.

In this study, molecular markers were developed based on PCR and real-time PCR combined with MLP-like gene for detection of Chunpoong in ginseng samples. The results showed 100% sensitivity. The InDel marker will be better used to differentiate between cross-cultivated products of *P. ginseng* and *P. quinquefolius*, while the SNP marker will be better used to analyze a Chunpoong population or a plant breeding program based on real-time PCR.

**Acknowledgements** This study was supported by the Korean Ginseng Center for Most Valuable Products (KGCMVP) of the Technology Development Program of Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

**REFERENCES**