Identification and Expression Analysis of Chloroplast p-psbB Gene Differentially Expressed in Wild Ginseng

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Abstract
Panax ginseng is a well-known herbal medicine in traditional Asian medicine. Although wild ginseng is widely accepted to be more active than cultivated ginseng in chemoprevention, little has actually been reported on the difference between wild ginseng and cultivated ginseng. Using suppressive subtraction hybridization, we cloned the p-psbB gene as a candidate target gene for a wild ginseng-specific gene. Here, we report that one of the clones isolated in this screen was the chloroplast p-psbB gene, a chlorophyll a-binding inner antenna protein in the photosystem II complex, located in the lipid matrix of the thylakoid membrane. Real-time results showed that the expression of the p-psbB gene was significantly up-regulated in wild ginseng as compared to cultivated ginseng. Thus, the p-psbB gene may be one of the important markers of wild ginseng.

1. Introduction
Ginseng, Panax ginseng C.A. Meyer, has been a commonly used herbal medicine in oriental countries, including China, Japan and Korea, for thousands of years. Ginseng is a deciduous perennial plant that belongs to the Araliaceae family. Currently, twelve species have been identified in the genus Panax. Ginseng is one of the most widely used herbal medicines in the world, which benefits to general health, including positive effects on the endocrine, cardiovascular, immune, and central nervous systems and preventing fatigue, oxidative damage, mutagenicity and cancer [1-5].

Cultivated ginseng is cultivated artificially and accounts for the majority of ginseng in the current market. Mountain wild ginseng grows in natural environments, vegetating in deep mountains, while mountain cultivated wild ginseng is seeded and grown in forests and mountains and is considered as a mimicry of mountain wild ginseng. And they have been shown to contain higher levels of ginsenosides. On the other hand, the reported differences in total ginsenoside contents between wild and cultivated ginseng were minimal [6-7]. In both Korea and China, wild ginseng is widely accepted to be more active than cultivated ginseng in chemoprevention. However, because of its high cost and sparse distribution, few systematic studies on wild ginseng have been done, and little has actually been reported on the differences between wild ginseng and other types of ginseng. Also, the lack of quality control has led to chaos in market distribution [8-9]. In addition, we searched for another novel gene that wild ginseng to be distinguished from cultivated ginseng, and found the p-psbB gene, which
2. Materials and methods

2.1. Various ginsengs for RNA isolation

The cultivated ginsengs (CGs) used in this experiment were 4 and 6 years of age and from various region in Korea. The wild ginsengs (WGs) used in this experiment were collected from Changbai Mt. in 2008, and were about 20 to 40 cm long, with masses of about 20 to 30 g and approximate ages of 30 to 50 years (Fig. 1). At the heart of this complex is the reaction center consisting of the D1 and the D2 proteins, where primary charge separation occurs [13]. Closely associated with the D1 and the D2 proteins are two similar chlorophyll a-binding proteins, CP43 and CP47 (product of the p-psbB gene) [14]. These proteins serve as an “inner antennae” system that is linked to a secondary light-harvesting system.

Here, we have cloned the p-psbB gene encoding CP47, a chlorophyll -binding inner antenna protein, as a candidate target gene of the wild ginseng-specific genes using suppressive subtraction hybridization (SSH). We have further analyzed the differentially expressed levels of the p-psbB gene between cultivated ginseng and mountain cultivated wild ginseng by means of real-time quantitative PCR.

Table 1 Primer for RT-PCR.

| Gene  | Primer sequence | Product size (bp) |
|-------|-----------------|-------------------|
| 18s   | F: 5’-AAC GAG ACC TCA GCC TGC TA-3’ | 187               |
|       | R: 5’-CCT GTC GGC CAA GGT TAT AG-3’ |                   |
| p-psbB| F: 5’-TGT CTT AAC GAG CCG GAA TC-3’ | 246               |
|       | R: 5’-TGT CTT AAC GAG CCG GAA TC-3’ |                   |

2.2. Total RNA isolation and mRNA purification

Ginseng was ground in liquid nitrogen by using a mortar and pestle, and RNA was isolated using the RNeasy Plant RNA Isolation Kit (Qiagen). The concentration of isolated RNA was estimated by measuring its absorbance at 260 nm. An aliquot of the RNA extract was treated with DNase-I (Invitrogen) prior to cDNA synthesis by using Superscript III reverse transcriptase (Invitrogen) and random hexamers according to the manufacturer’s protocol.

2.3 Suppressive subtractive hybridization

Suppressive subtractive hybridization (SSH) was performed using Clontech PCR-SelectTM cDNA Subtraction Kit (Clontech) according to the manufacturer’s protocol. SSH method includes several steps (cDNA synthesis, RsaI digestion and adaptor ligation, two rounds of hybridization and PCR) for isolating differentially expressed genes.

The CDNA fragments, derived from SSH forward subtractive library (tester: mountain cultivated wild ginseng; driver: ginseng), were cloned into pEC-T vector (KOMA Co., Seoul, Korea). The positive clones containing inserted fragments were identified by using the colony-PCR method.

2.4. RT-PCR Assay

Semi-quantitative RT-PCR was performed to compare the differential expression of the genes in the SSH library by using gene-specific primers. Total RNA (2 µg) was used for cDNA synthesis according to the First Strand cDNA Synthesis Kit (Invitrogen), and 1.0 µl of cDNAs was used as a template for PCR. PCR amplification was performed under the following conditions: 95°C for 5 min, 30 cycles at 95°C for 45 s, 54°C for 30 s, and 72°C for 60 s. The final incubation was done at 72°C for 5 min. PCR products were electrophoresed in a 2% agarose gel.

2.5. Quantitative Real-time quantitative RT-PCR

Real-time quantitative RT-PCR detection was performed with a StepOne machine and Fast SYBR Green Master Mix (Applied Biosystem, USA) and were measured in a 96-well plate. For each well, the 20 µl reaction involved 10 µl of the 2 X Fast SYBR Green Master Mix, 0.5 µM each of forward and reverse primer, 2.75 µl of DNase-free Hi-O, 2 µl of cDNA templates. PCR reactions were performed using the following parameters: 8 min at 95°C and 40 cycles of 45 s at 95°C, 45 s at 54°C and 45 s at 72°C. PCR products were melted by gradually increasing the temperature from 60°C to 95°C in 0.5°C steps.

The identities of the amplicons and the specificity of the reaction were verified by using a melting curve analysis. Normalization of the cDNA templates was achieved by using 18S quantification. The primers presented in Table 1 were used to analyze p-psbB gene expressions.

2.6. Sequencing and homology analysis

PCR products were cloned into the pEC-T vector (KOMA Co., Ltd, Seoul, Korea) and then sequenced by using the ABI 3700 DNA sequencers (PerkinElmer Applied Biosystems). The
sequence analysis was performed using Chromas sequence analysis software. BLASTn was used to study similar nucleotide sequences.

3. Results

3.1. Isolation of differentially expressed genes in wild ginseng

To identify wild ginseng-specific genes, wild ginseng cDNAs were subtracted from a pool of cultivated-ginseng cDNAs (Fig. 1). The subtraction was expected to significantly reduce common cDNAs and to enrich for wild-ginseng-specific cDNAs. More than 100 transformants were obtained from the library, and the recombinant efficiency detected by using colony-PCR was about 90%.

One hundred positive clones confirmed by PCR amplification were randomly selected, from which, 16 significantly different clones were sequenced. Because the suppression subtractive hybridization procedure includes a restriction enzyme digestion of the cDNAs produced, none of the clones obtained from the resulting libraries were full length.

Among the novel cDNAs identified here as putative wild-ginseng-specific genes is a putative chloroplast p-psbB, designated as p-psbB (Fig. 2). The open reading frame contained in the p-psbB cDNA encodes a protein with 509 amino acids with a predicted molecular mass of 56,364 Da (Fig. 3).

3.2. RT-PCR analysis

To confirm the differential expression of the p-psbB gene, we employed the RT-PCR analysis was employed. Total cellular RNA from the four cultivated ginsengs, and two mountain wild ginsengs were used for the RT-PCR analysis. The p-psbB gene-specific primers were designed to amplify both the cDNA from the cultivated ginsengs and from the wild ginsengs. The number of PCR cycles was optimized to ensure that the comparison of the levels of expressions of the p-psbB gene was within the linear phase of amplification.

As shown in Fig. 4, all of the p-psbB transcripts derived from mountain wild ginsengs reveal an upper band whereas all of the p-psbB transcripts derived from the cultivated ginsengs showed lower bands. Thus, these results suggest that p-psbB mRNA is specifically expressed in wild ginsengs.

3.3. Real-time RT-PCR analysis

To further verify that the p-psbB gene is differentially expressed between cultivated and wild ginsengs, we performed quantitative real-time PCR. Results showed that the relative transcription levels of p-psbB were significantly up-regulated in wild ginseng (p < 0.05), the levels of p-psbB transcripts in cultivated ginsengs being nearly undetectable (Fig. 5). Taken together, these results suggest that the p-psbB gene showed high levels of differential expression in wild ginseng (Fig. 6).
prior to creating the library and has the advantage that reverse transcriptions are amplified efficiently [16].

We isolated a novel gene, p-psbB ([Panax ginseng chloroplast p-psbB]). Sequence analysis revealed that p-psbB possessed significant homology to p-psbB sequences reported from other plant species. p-psbB mRNA is differentially expressed in wild ginsengs. Thus, p-psbB may be one of the important markers of wild ginseng.

The p-psbB encoded CP47 protein, a chlorophyll binding inner antenna protein in the photosystem II complex is located in the lipid matrix of the thylakoid membrane. The p-psbB has a light-harvesting function; it absorbs light and transfers the excitation energy to the reaction center of photosystem II [14]. Even more importantly, it also accepts excitation energy from the peripheral antenna and transfers it to the reaction center as well. However, although the mechanism by which p-psbB is up-regulated in wild ginseng is not clear, we suppose that it may an important marker of wild ginseng.

4. Discussion

P. ginseng is categorized as either cultivated (in the farm) or wild (in the mountain) according to its different nurturing methods. Cultivated ginseng is systematically farmed on an open land and is harvested after a 4 to 6 year of cultivation period. On the other hand, wild ginseng is planted through seeding in a deep mountain. Wild ginseng is slower in growth and more sensitive to environmental changes than cultivated ginseng, showing a preference for areas with fluctuating daily temperatures and less exposure to direct sunlight. These differences may result in a variation of active compounds between cultivated and wild ginseng. In both Korea and China, wild ginseng is widely accepted to produce more potent pharmacological activities between wild and cultivated ginseng. However, few studies have been conducted to compare the food components and medicinal activity than cultivated ginseng. However, although the mechanism by which p-psbB is up-regulated in wild ginseng is not clear, we suppose that it may an important marker of wild ginseng.

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