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Original Article

Identification and Analysis of the Chloroplast *rpoC1* Gene Differentially Expressed in Wild Ginseng

Kwang-Ho Lee[1], Ki-Rok Kwon[2]*, Won-Mo Kang[3], Eun-Mi Jeon[3], Jun-Hyeog Jang[3]

[1]Department of Acupuncture & Moxibustion, Sangji University College of Oriental Medicine, Wonju, Korea
[2]Research Center of the Korean Pharmacopuncture Institute, Seoul, Korea
[3]Department of Biochemistry, Graduate School of Medicine, Inha University, Incheon, Korea

Key Words

Panax ginseng; rpoC1 gene; wild ginseng; suppressive subtraction hybridization (SSH); markers of wild ginseng

Abstract

Panax ginseng is a well-known herbal medicine in traditional Asian medicine, and wild ginseng is widely accepted to be more active than cultivated ginseng in chemoprevention. However, little has actually been reported on the difference between wild ginseng and cultivated ginseng. Thus, to identify and analyze those differences, we used suppressive subtraction hybridization (SSH) sequences with microarrays, real-time polymerase chain reaction (PCR), and reverse transcription PCRs (RT-PCRs). One of the clones isolated in this research was the chloroplast *rpoC1* gene, a β subunit of RNA polymerase. Real-time RT-PCR results showed that the expression of the *rpoC1* gene was significantly upregulated in wild ginseng as compared to cultivated ginseng, so, we conclude that the *rpoC1* gene may be one of the important markers of wild ginseng.

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1. Introduction

Ginseng (*Panax ginseng* C.A. Meyer) has been commonly used as a herbal medicine in oriental countries, including China, Japan and Korea, for thousands of years. The herbal root is named ginseng because it is shaped like a man. Ginseng is a deciduous perennial plant that belongs to the *Araliaceae* family and currently, twelve species have been identified in the genus *Panax.* In addition, ginseng is one of the most widely used herbal medicines in the world, and benefits general health, by having positive effects on the endocrine, cardiovascular, immune, and central nervous systems, and by preventing fatigue, oxidative damage, mutagenicity and cancer [1-3].

Cultivated ginseng (CG) is cultivated artificially and accounts for the majority of ginseng in the current market. Mountain wild ginseng (WG) grows in natural environments, vegetating in deep mountains, and mountain cultivated ginseng (MCG) can be considered as a mimicry of WG as it is seeded and grown in forests and mountains. WG is considered to be superior to CG, and it has been shown to contain higher levels of ginsenoside, although the reported differences in the total ginsenoside content between WG and CG [4]. Ginsenoside levels were consistently lower in ginseng grown in more intensively cultivated gardens, but growth was consistently higher [5]. In both Korea and China, WG is widely accepted to be more active than CG in chemoprevention. However, little has actually been reported on the differences between WG and CG. Also, the lack of quality control has led to chaos in market distribution [6-7]. Thus, our research team conducted a study to identify WG specific genes for standardization, and we succeeded inidentifying a novel clone, the NRT2 gene, that is a high-affinity nitrate transporter [8].

The technique of suppressive subtractive hybridization

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This paper meets the requirements of KS X ISO 9706, ISO 9706-1994 and ANSI/NISO Z39.48-1992 (Permanence of Paper).

*Corresponding author
Ki-Rok Kwon. Korean Pharmacopuncture Institute, 4F, Association of Korean Oriental Medicine B/D, 26-27, Gayang-dong, Gangseo-gu, Seoul 157-200, Korea.
Tel: +82-2-2658-9137
E-mail: beevenom@paran.com

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(SSH) is believed to generate an equalized representation of differentially expressed genes and to provide a high enrichment of differentially expressed mRNA [9]. SSH overcomes the limitations of other gene analysis methods for differential expression. Its polymerase chain reaction (PCR)-based approach allows for the effective removal of common genes from the RNA population prior to creating the library and has the advantage that reverse transcriptions are amplified efficiently [10]. Chloroplast genomes from several plant species have been sequenced, and have revealed *rpoA, rpoB* and *rpoC* genes, which encode proteins homologous to the α, β and β' subunits of bacterial RNA polymerase (*rpoC* is usually split into *rpoC1* and *rpoC2*, which encode β' and β'' subunits). Soluble multi-subunit RNA polymerases have been purified from chloroplasts of several plant species and often include 7-13 subunits, some of which can be identified as products of the *rpoA-rpoC* genes [11]. In this study, the differentially expressed *p-rpoC1* gene was identified, and the differences between CG and WG were analyzed using SSH sequences with microarrays, real-time PCRs, and reverse transcription PCRs (RT-PCR).

2. Materials and methods

2.1. Various ginsengs for RNA isolation

The CGs used in this experiment were 6 years of age and were from various regions in Korea. The WGs were 20 to 40 cm long with masses of 20-30 g and age of 30~50 years. These samples were collected from Changbai Mountain in 2008.

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 (SSH)

Suppressive subtractive hybridization (SSH) was performed using the Clontech PCR-SelectTM cDNA Subtraction Kit (Clontech) according to the manufacturer' s protocol. The SSH method includes six steps (cDNA synthesis, RsaI digestion and adaptor ligation, two rounds of hybridization and two types of PCRs) for isolating differentially expressed genes. The cDNA fragments, derived from the SSH forward subtractive library (tester: WG; driver: CG), were cloned in pEC-T vector (KOMA Co., Seoul, Korea). The positive clones containing inserted fragments were identified by using the colony-PCR method.

2.4. Quantitative real-time RT-PCR

Real-time quantitative RT-PCRs detection was detected with an StepOne machine and Fast SYBR Green Master Mix (Applied Biosystem, USA) and were measured in a 96-well plate. For each well, a 20 $\mu\ell$ reaction involved 10 $\mu\ell$ of the 2 X Fast SYBR Green Master Mix, 0.5 M each of forward and reverse primer, 2.75 $\mu\ell$ of DNase-free H_2O and 2 $\mu\ell$ of cDNA templates. PCR reactions were performed using the following parameters: 8 min at 95℃ and 40 cycles each of 45 s at 95℃, 45 s at 56℃ and 45 s at 72℃. PCR products were melted by gradually increasing the temperature from 60 to 95℃ in 0.5℃ 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-rpoC1* gene expression.

Table 1 Primer for the RT-PCR

Gene	Primer sequence	Product size (bp)
18s	F: 5'-AAC GAG ACC TCA GCC TGC TA-3' R: 5'-CCT GTC GGC CAA GGT TAT AG-3'	187
p-rpoC1	F: 5'-TGT CTT AAC GAG CGG GAA TC-3' R: 5'-TGT CTT AAC GAG CGG GAA TC-3'	203

2.5. RT-PCR Assay

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

2.6. Sequencing and homology analysis

PCR products were cloned in the pEC-T vector (KOMA Co., Ltd, Seoul, Korea) and were then sequenced by using ABI 3700 DNA sequencers (Perkin Space Elmer 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 WG-specific genes, we subtracted WG cDNAs from a pool of CG cDNAs. The subtraction was expected to significantly reduce common cDNAs and to enrich WG-specific cDNAs. More than 100 tranformants were obtained from the library, and the recombinant efficiency detected by using colony-PCR was about 90%. One hundred positive clones that had been confirmed by using PCR amplification were randomly selected, from which, 16 significantly different clones were sequenced. Because the SSH procedure includes a restricted 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 wildginseng-specific gene was the chloroplast *rpoC1*, designated as *p-rpoC1* (Fig.1). The open reading frame contained in the *p-rpoC1* cDNA encodes a protein with 690 amino acids with a predicted molecular mass of 79,394 Da (Fig. 2).

AAGATTCATAGTGGACTTCGGGGGGGGCTTCTTTTGCAGAAATAACGC
GTTGATCTAGTCGCCACCGAAGCCACAAAGGACTATCTAAATTGATTCG
TTTCTGCCGATAAGCTCCAATTGCATCATAGGAATTACAAAAAAAGGGT
TCTTTTTTTTTCGTACACTTATAGTTATTATCGTAAATTCTTTCATTTTGA
TAGTTTCTGCGATTACATGGATTATACCTATTTACACAAATACCCCGAC
GATTCCCGCTCGTTAAGACATAGAGTCCAATAAGCATATCTTGAGT

Figure 1 Determined DNA sequence of the putative *Panax ginseng rpoC1* gene.

Figure 2 Nucleotide and predicted amino acid sequences of the *Panax ginseng rpoC1* gene The deduced amino acid sequence of the *rpoC1* gene is shown in one-letter symbols below the nucleotide sequence. Amino acid residues are numbered beginning with the first methionine, and the translation termination codon is denoted by an *asterisk. Numbers* to the *right* of the sequence correspond to amino acids (*lower*) and nucleotides (*upper*).

3.2. RT-PCR analysis

To confirm the differential expression of the *rpoC1* gene, we employed a RT-PCR analysis. Total cellular RNAs from the four CGs and two WGs were used for the RT-PCR analysis. *p-rpoC1* gene-specific primers were designed to amplify the cDNA from both the CGs and the WGs. The PCR cycles were optimized to ensure that comparisons of the levels of expressions of the *p-rpoC1* gene would be within the linear phase of amplification.

As shown in Fig. 3, all of the *p-rpoC1* transcripts derived from the WGs revealed upper bands whereas all of the *rpoC1* transcripts derived from the CGs showed lower bands. Taken together, these results suggest that *p-rpoC1* mRNA is specifically expressed in WG.

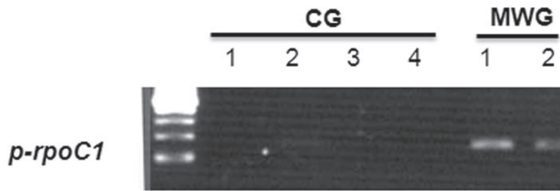

Figure 3 RT-PCR analysis of the differential expression of *p-rpoC1* genes Total RNAs (2.0 g) from four cultivated ginsengs (Ganghwa 4 and 6 years and Pongki 4 and 6 years) and two mountain wild ginsengs (WG) were used for the RT-PCR by using the specific primers *p-rpoC1*.

3.3. Real-time RT-PCR analysis

To further verify that the *p-rpoC1* gene is differentially expressed between CG and WG, we performed a quantitative real-time PCR. Results showed that the relative transcription levels of *p-rpoC1* were significantly up-regulated in WG ($p > 0.05$) and that the levels of *p-rpoC1* transcripts in CG were nearly undetectable (Table 2). Taken together, these results suggest that the *p-rpoC1* gene shows a high level of differential expression in WG.

Table 2 Quantitative real-time RT-PCR analysis of *p-rpoC1* transcripts

Sample	p-rpoC1 gene
CG(Ganghwa 4y)	3.9 ± 0.22
CG(Ganghwa 6y)	5.6 ± 0.49
CG(Pongki 4y)	0.4 ± 0.13
CG(Pongki 6y)	1.2 ± 0.34
WG	541.1 ± 56.32
WG	135.6 ± 32.54

4. Discussion

Panax ginseng is categorized as either cultivated or wild according to its different nurturing methods. CG is systematically farmed on open land and is harvested after a 5 to 6 year of cultivation period. On the other hand, WG is grown in natural environment in a deep mountains. WG is slower in growth and more sensitive to environmental changes than CG, 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 CG and WG. In both Korea and China, WG is widely accepted to produce more potent medicinal activity than CG. However, few studies have been conducted to compare the food components and pharmacological activities between WG and CG.

In the present study, to identify a WG-specific gene, we subtracted cDNAs expressed in WGs from those in CGs by using the SSH technique [9]. We isolated a novel gene, *p-rpoC1* (Panax ginseng chloroplast *rpoC1*), that encoded the ribonucleic acid (RNA) polymerase core subunit. Sequence analysis revealed that *p-rpoC1* possessed significant homology to the rpoC1 sequences reported from other plant species. *p-rpoC1* mRNA is specifically expressed in WG.

The *rpoC* gene encodes the β-subunit of RNA polymerase in a chloroplast (Fig. 4). Plastid genes in photosynthetic higher plants are transcribed by at least two RNA polymerases. The plastid *rpoA, rpoB, rpoC1*, and *rpoC2* genes encode subunits of the plastid-encoded plastid RNA polymerase (PEP), an *Escherichia coli-like* core enzyme [12]. The second enzyme is referred to as the nucleus-encoded plastid RNA polymerase (NEP), because its subunits are assumed to be encoded in the nucleus. The promoters for PEP are reminiscent of the *E.coli* σ^{70}-type promoters and have two conserved hexameric blocks of sequences (TTGACA or "-35" element; TATAAT or "10" element) 17 to 19 nucleotides apart. Transcription from PEP promoters initiates 5 to 7 nucleotides downstream of the "10" promoter element [13]. Recently, deletion of *rpoA, rpoB, rpoC1*, and *rpoC2* genes was found to yield photo-synthetically defective plants that lacked PEP activity while maintaining transcription specificity from NEP promoters, suggesting that the PEP β-subunit is essential for PEP transcription activity, but is not required for NEP transcription activity [9].

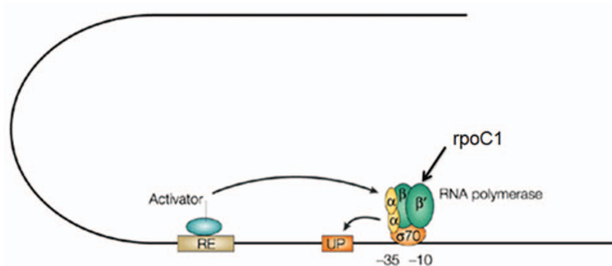

Figure 4 Schematic diagram of the *rpoC1* subunit in RNA polymerase

In conclusion, although these observations suggest that the *p-rpoC1* gene is specifically expressed in WG, little is known about how the differentially expressed *p-rpoC1* influences the WG. Nevertheless, our study suggests that *p-rpoC1* may be one of the important markers of WG.

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