

# Identification and Analysis of the Novel *pGAPDH-w* Gene Differentially Expressed in Wild Ginseng

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## Key Words

hybridization (SSH), cultivated ginseng, PCR, *pGAPDH-w* gene, suppressive subtraction, wild ginseng

## Abstract

**Objective:** *Panax ginseng* is one of the most medicinally used herbal medicines in the world. Wild ginseng is widely accepted to be more active than cultivated ginseng in chemoprevention. However, little has actually been reported on the differences between wild ginseng and cultivated ginseng.

**Method:** To identify wild ginseng-specific genes, we used suppressive subtraction hybridization.

**Results:** We report that one of the clones isolated in this screen was the *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) gene (designated *pGAPDH-w*). DNA BLAST sequence analysis revealed that this *pGAPDH-w* gene contained novel sequences of 94 bp. RT-PCR results showed that the expression of the *pGAPDH-w* gene was significantly up-regulated in the wild ginseng as compared with the cultivated ginseng.

**Conclusion:** The *pGAPDH-w* gene may be one of the important markers of wild ginseng.

Received: Apr 4, 2012 Accepted: Oct 31, 2012

## 1. Introduction

Ginseng, *Panax ginseng* C.A. Meyer, has been commonly used herbal medicine in oriental countries including China, Japan and Korea for thousands of years [1]. The genus name "*Panax*" is derived from Greek, and means cure all. 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. Currently, twelve species have been identified in the genus *Panax*. Among them, *Panax ginseng* C. A. Meyer (Asian ginseng), *Panax quinquefolium* L. (American ginseng), and *Panax notoginseng* (Burkill) F. H. Chen (notoginseng) are most important herbs used for different medical conditions [2]. The most prominent constituents of ginseng are saponins, generally called ginsenosides, which are emphasized in cancer chemoprevention and therapeutics [3-4].

In both Korea and China, wild ginseng (WG) widely accepted to be active than cultivated ginseng (CG) in chemoprevention. However, their high costs and rare distributions, few studies have reported on the difference between WG and CG. Also, lack of quality control has led to chaos in market distribution [5]. Thus, our research team conducted a study to identify WG specific genes for standardization, and we succeeded in identifying some WG novel clones, the

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*NRT2*, *psbB* and *rpoC1* genes [6-8]. For probable conquest through scientific and systematic methods, we attempted to find other differentially expressed genes to identify a WG-specific gene. For that we used a suppression subtractive hybridization (SSH) technique cDNA expressed in WG from that expressed in CGs [9].

The SSH technique is believed to generate an equalized representation of differentially-expressed genes and to provide a high enrichment of differentially expressed mRNA. 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 of efficiently amplified reverse transcriptions

[10]. In this study, we identified the differentially-expressed gene *pGAPDH-w* and analyzed the difference between CG and WG.

## 2. Materials and methods

### 2.1. Various Ginsengs for RNA isolation

The CGs used in this experiment were 4 and 6 yr of age from various regions in Korea. The WGs used in this experiment was collected from Changbai mountain, in 2008. They were about 20-40 cm long, weighed about 20-30 g with the approximate ages of 30-50 yr (Fig. 1). The authenticity of the WGs were examined by a panel of the Korean medicine experts.



**Figure 1** Ganghwa cultivated ginseng (A), Guemsan cultivated ginseng (B) and wild mountain ginsengs used for RNA isolation (C-E).

## 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 an RNeasy Plant RNA Isolation kit (Qiagen). The concentration of the 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)

SSH was performed using a Clontech PCR-Select™ 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 PCR) for isolating differentially expressed genes. The cDNA fragments derived from the SSH forward subtractive library (tester: wild ginseng; driver: cultivated ginseng) were cloned into pEC-T vector (KOMA). The positive clones containing inserted fragments were identified by using the colony-PCR method.

## 2.4. Semi-quantitative real time-PCR

Semi-quantitative real time (RT)-PCR was performed to compare the differential expression of the genes in the SSH library by using gene specific primers. Total RNA (2 g) was used for cDNA synthesis with a First Strand cDNA Synthesis Kit (Invitrogen), and 1.0  $\mu$ l of cDNAs were 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. Real-time quantitative-PCR

Real-time quantitative-PCR detection was performed with a StepOne machine and Fast SYBR Green Master Mix (Applied Biosystem, USA), and was measured in a 96-well

plate by using a StepOne RT-PCR system (Applied Biosystems). For each well, the 20  $\mu$ l reaction involved 10  $\mu$ l of 2X Fast SYBR Green Master Mix, 0.5  $\mu$ l each for the forward and the reverse primers, 2.75  $\mu$ l of DNase-free H<sub>2</sub>O and 2  $\mu$ l 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 56°C and 45 s at 72°C. PCR products were melted by gradually increasing the temperature from 60 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 an 18S quantification. The primers presented in Table 1 were used to analyze of *pGAPDH-w* gene expressions.

## 2.6. Sequencing and homology analysis

PCR products were cloned into the pEC-T vector (KOMA) and were then sequenced by the ABI 3700 DNA sequencers (Perkin Elmer Applied Biosystems). The sequence analysis was performed using Chromas sequence analysis software. BLAST was used for the study of similar nucleotide sequences.

## 3. Results

### 3.1. Isolation of differentially-expressed genes in wild ginseng

To identify WG-specific genes, we subtracted WG cDNA from a pool of CG cDNA. The subtraction was expected to significantly reduce common cDNA and enrich for WG-specific cDNA. More than 100 transformants were obtained from the library, and the recombinant efficiency detected by 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 SSH procedure includes a restricted enzyme digestion of the cDNA produced, none of the clones obtained from the resulting libraries were full length.

**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'	
<i>pGAPDH-w</i>	F: 5'-GGT GGT GCA AAG AAG GTT GT-3'	203
	R: 5'-CAA CCT GCA AAA TTT TAA GAA GA-3'	

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CCTGGTGGTGAGACAAAGGTTGTGATTTCTGCGCCTAGCAAGGATGCACCCATGTTTGTGTTGGGT
GTCAATGAGAAGGAATACAAGCCTGAACTTGACATTGTCTCTAATGCTAGCTGCACTACCAATTGT
CTTGCTCCTCTGGCCAAGGTTACATGAACTCCTTTTCAAATACAAGTATATACTGGCATTGTTGAT
ATATAAATTCAATAACCTCTGGTCTTTCTTCTTAAAATTTTGCAGGTTGTTAATGATAGATTTGGCA
TTGTTGAGGGGCTCATGACAGGGGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTC
GGTACCAAGCTTGGCGTAATCATGGTCATAGCCTGGTTTCCAG

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**Figure 2** Determined DNA sequence of the wild ginseng *pGAPDH-w* gene. Novel sequences are in red.

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(New)
pGAPDH-w AGCTGCACTACCAATTGTCTTGCTCCTCTGGCCAAGGTTACATGAACTCCTTTTCAAATACAAGTATATACTGGCATTGTTGATATATAAATT
pGAPDH   AGTTGCACTACCAACTGTCT TGCTCCCCTTGCAAAGGTGA-----
(Well known)
          CAATAACCTCTGGTCTTTCTTCTTAAAATTTTGCAGGTTGTTAATGATAGATTTGGCATTGTTGAGGGGCTCATGAC
          ----- TTAACGATAGGTTTGGCATTGTTGAGGGGCTCATGAC

```

**Figure 3** DNA sequence alignment of the *pGAPDH-w* gene with known *pGAPDH* genes across the species. Novel sequences are in red.

Among the cDNAs identified here as putative wild-ginseng-specific gene is a putative homology of glyceraldehyde-3-phosphate dehydrogenases (*GAPDH*), designated *pGAPDH-w* (Fig. 2). Searches of the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) revealed significant homology (85%) to panax *GAPDH* sequences. Interestingly, the DNA BLAST sequence analysis revealed that this *pGAPDH-w* gene contained novel sequences of 94 bp (Fig. 3).

### 3.2 Quantitative RT-PCR analysis

To further verify that the *pGAPDH-w* gene is differentially expressed between CGs and WGs, quantitative RT-PCR was performed. Results showed that the relative transcription levels of the *pGAPDH-w* gene were up-regulated in WGs, and the levels of the *pGAPDH-w* transcripts in CGs were nearly undetectable (Fig. 4). Taken together, these results suggest that the *pGAPDH-w* gene showed high levels of differential expression in WG.

### 3.3. RT-PCR Analysis

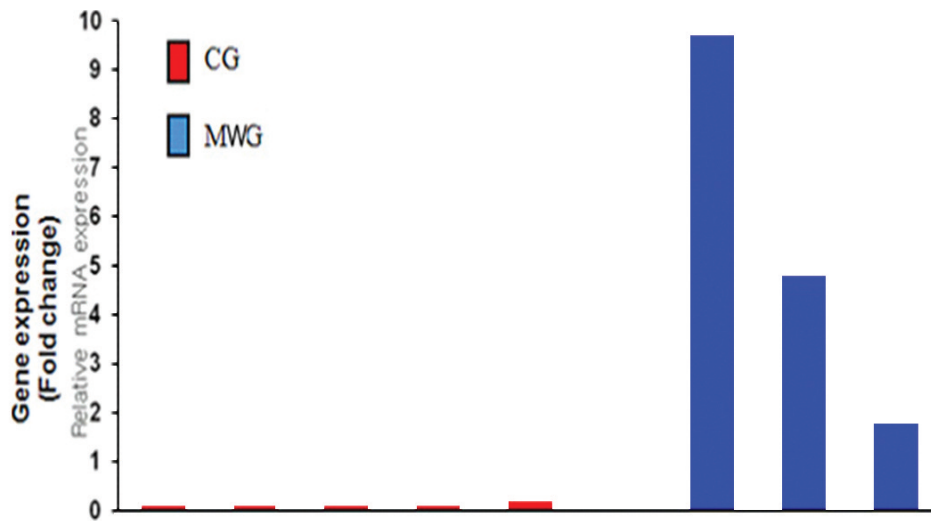
To confirm the differential expression of the *pGAPDH-w*

genes, we employed a RT-PCR analysis, the total cellular RNA from the five CGs, and three WGs were used for the RT-PCR analysis. *pGAPDH* gene-specific primers were designed to amplify both the cDNA from the CG and the WGs. The number of PCR cycle was optimized to allow comparisons of the expression of the *pGAPDH* gene the linear phase of amplification.

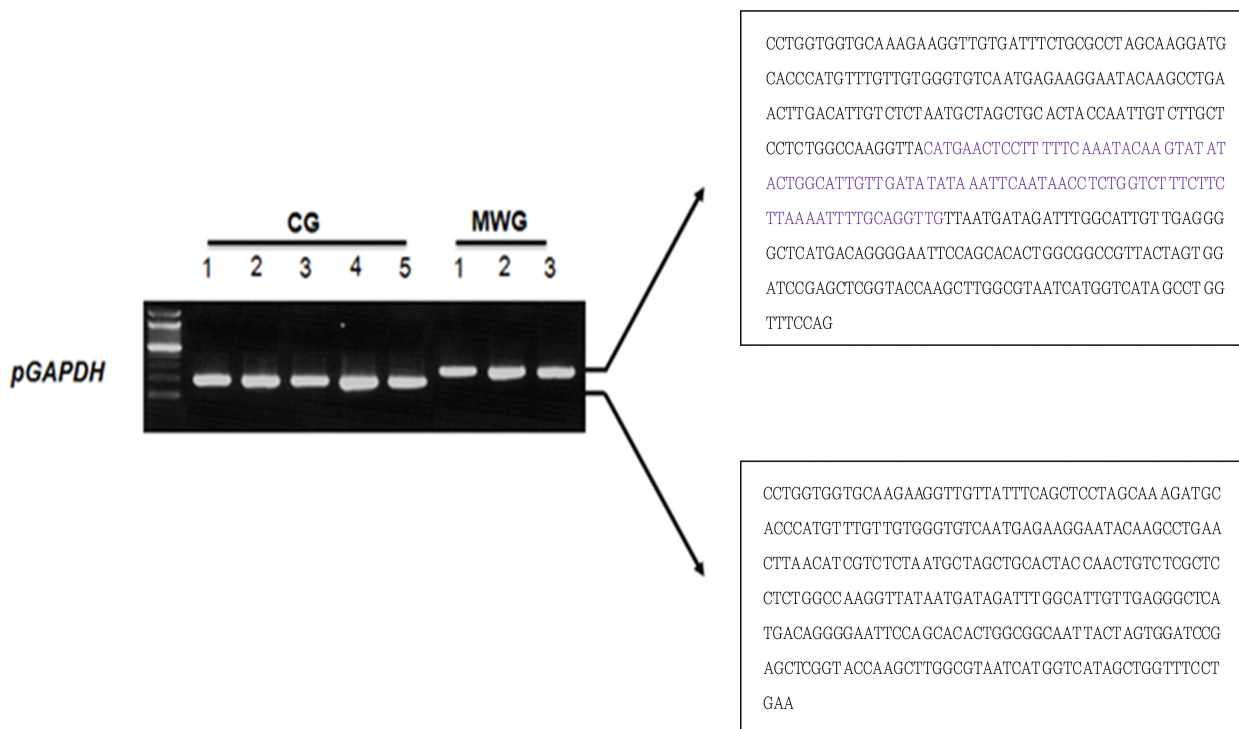
As shown in Fig. 5, all of the *p-GAPDH* transcripts derived from WGs revealed band, whereas all of the *p-GAPDH* transcripts derived from the CG showed no bands. The difference between the upper bands and lower bands corresponds with the 94 bp novel sequence that is frequently found in WGs. Taken together, these results suggest that the *pGAPDH-w* mRNA is specifically expressed in WGs.

## 4. Discussion

*Panax ginseng* is categorized as either cultivated or wild according to different nurturing methods. CG is systematically farmed on an open land and is harvested after a 5-6 yr of cultivation. On the other hand, WG grows



**Figure 4** Quantitative real time-PCR analysis of *pGAPDH-w* transcripts. Total RNA extracted from the cells (2  $\mu$ g) was reverse-transcribed to cDNA (40  $\mu$ l), and aliquots (1  $\mu$ l) were applied to real-time PCR (20  $\mu$ l) with each primer (0.4 mM). Values represent relative expressions of the *pGAPDH-w* gene calculated using the threshold cycle number (CT) of three mountain wild ginsengs (MWGs) compared with that of five cultivated ginsengs (CGs). Each value was adjusted using the CT of internal control (18 s).



**Figure 5** RT-PCR analysis of differential expressions of the *pGAPDH-w* genes. Total amount of RNA (2.0 g) from five cultivated ginsengs (CGs) and three mountain wild ginsengs (MWGs) were used for RT-PCR by using the specific primers *pGAPDH*. The 5' forward and the 3' reverse-complement PCR primers for amplification of *pGAPDH* sequences were 5'-GGT GGT GCA AAG AAG GTT GT-3' and 5'-GTC ATG AGC CCC TCA ACA AT-3', respectively.

naturally in a deep mountain. 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.

Based on the growth environment and the cultivation method, commercially-trade ginseng is classified into three grades of ginseng, CG, mountain cultivated ginseng and WG. CG is cultivated artificially on farms and account for the majority of ginseng in the current market. MG grows in natural environments, vegetating in deep mountains, and mountain cultivated ginseng can be considered as a mimicry of WG which is seeded and grows up in forests and mountains. WG is considered superior to CG, and it has been shown to contain higher levels of ginsenosides, although Lui and Staba reported minimal differences in total ginsenoside content between WG and CG [11]. Compared to those of WG, the ginsenoside levels of CGs from more intensively cultivated garden locations were consistently lower, but the growth rate was consistently higher [12].

Most of the pharmacological actions of ginseng are attributed to a variety of ginsenosides, which are triterpenoid saponins [13, 14]. The physiological and the medicinal effects of the various ginsenosides are different [15]. These differences may result in variations of the 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 components and the pharmacological activities between WG and CG.

In the present study, to identify the WG specific gene, we subtracted the cDNA expressed in WGs from that expressed in CG by using the suppression subtractive hybridization (SSH) technique, and we isolated a gene, the *pGAPDH-w* (wild *Panax ginseng* *GAPDH* gene). Sequence analysis revealed that the *pGAPDH-w* possessed significant homology to the *GAPDH* sequences reported from other plant species. However, *pGAPDH-w* gene had an additional 94 bp compared to known the *pGAPDH*, which is expected to be a unique sequence in WG. The *pGAPDH-w* mRNA is specifically expressed in WG ( $P < 0.05$ ). Thus, the *pGAPDH-w* gene may be one of the important markers of WG. Higher plants contain two separate phosphorylating glyceraldehyde-3-phosphate dehydrogenases. One species, the classic enzyme of glycolysis (EC 1.2.1.12), is NAD-specific and is mainly located in the cytoplasm. The other species (EC 1.2.1.13) is active with NADP (H), but can also use NAD (H) as a coenzyme [16, 17]. It is a marker enzyme of chloroplasts and functions in the photosynthetic Calvin cycle. Although these observations suggest that the novel 94 bp sequence from the *pGAPDH-w* gene is specifically expressed in WG, its precise structure and function needs further

investigation.

## Acknowledgments

This study was supported by the Technology Development Program for Agriculture and Forestry (108069-03-1-CG000), Ministry for Agriculture, Forestry and Fisheries, Republic of Korea.

## References

1. Kwon KR, Owi JS, Kim SW. [Literature study of wild ginseng]. *Pharmacopuncture*. 2003;6(2):67-75. Korean.
2. Wang CZ, Mehendale SR, Yuan CS. Commonly used antioxidant botanicals: active constituents and their potential role in cardiovascular illness. *Am J Chin Med*. 2007;35(4):543-58.
3. Tachikawa E, Kudo K, Hasegawa H, Kashimoto T, Sasaki K, Miyazaki M, et al. In vitro inhibition of adrenal catecholamine secretion by steroidal metabolites of ginseng saponins. *Biochem Pharmacol*. 2003;66(11):2213-21.
4. Lee Y, Jin Y, Lim W, Ji S, Choi S, Jang S, et al. A ginsenoside-Rh1, a component of ginseng saponin, activates estrogen receptor in human breast carcinoma MCF-7 cells. *J Steroid Biochem Mol Biol*. 2003;84(4):463-8.
5. Kwon KR, Seo JC. [Genetical identification of Korean wild ginseng and American wild ginseng by using pyrosequencing method]. *Kor J Herbology*. 2004;19(4):45-50. Korean.
6. Kwon KR, Park WP, Kang WM, Jeon EY, Jang JH. Identification and analysis of differentially expressed genes in mountain cultivated ginseng and mountain wild ginseng. *J Acupunct Meridian Stud*. 2011;4(2): 123-8.
7. Kim DY, Kwon KR, Kang WM, Jeon EY, Jang JH. Identification and expression analysis of chloroplast *psbB* gene differentially expressed in wild ginseng. *Pharmacopuncture*. 2012;15(1):18-22.
8. Lee KH, Kwon KR, Kang WM, Jeon EM, Jang JH. Identification and analysis of the chloroplast *rpoC1* gene different expressed in wild ginseng. *Pharmacopuncture*. 2012;15(2): 20-3.
9. Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, et al. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA*. 1996;93(12):6025-30.
10. Diatchenko L, Lukyanov S, Lau YF, Siebert PD. Suppression subtractive hybridization: a versatile methods for identifying differentially expressed genes.

- Methods Enzymol. 1999;303:349-80.
11. Lui JH, Staba EJ. The ginsenosides of various ginseng plants and selected products. *J Nat Prod.* 1980;43(3):340-6.
  12. Lim W, Mudge KW, Vermeylen F. Effects of population, age, and cultivation methods on ginsenoside content of wild American ginseng (*Panax quinquefolium*). *J Agric Food Chem.* 2005;53(22):8498-505.
  13. Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. *Biochem Pharmacol.* 1999;58(11):1685-93.
  14. Huang YC, Chen CT, Chen SC, Lai PH, Liang HC, Chang Y, et al. A natural compound (ginsenoside Re) isolated from *Panax ginseng* as a novel angiogenic agent for tissue regeneration. *Pharm Res.* 2005;22(4):636-46.
  15. Sengupta S, Toh SA, Sellers LA, Skepper JN, Koolwijk P, Leung HW, et al. Modulating angiogenesis: the yin and the yang in ginseng. *Circulation.* 2004;110(10):1219-25.
  16. Cerff R. Glyceraldehyde-3-phosphate dehydrogenase (NADP) from *Sinapis alba* L. NAD(P)-induced conformation changes of the enzyme. *Eur J Biochem.* 1978;82(1):45-53.
  17. Cerff R. Glyceraldehyde-3-phosphate dehydrogenase (NADP) from *Sinapis alba* L: a reversible association of the enzyme with a protein factor as controlled by pyridine nucleotides in vitro. *Plant Physiol.* 1978;61(3):369-72.