

## Isolation and characterization of a novel short-chain alcohol dehydrogenase gene from *Panax ginseng*

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The cDNA of alcohol dehydrogenase (*PgADH*) was isolated and characterized from the leaf of *Panax ginseng*. The cDNA had an open reading frame of 801 bp and a deduced amino acid sequence of 266 residues. The calculated molecular mass of the mature protein is approximately 29 kDa with a predicted isoelectric point of 6.84. Homology analysis revealed that the deduced amino acid of *PgADH* shares a high degree of homology with the short-chain ADH proteins of other plants. Genomic DNA hybridization analysis indicated that *PgADH* represents a multi-gene family. The expression of *PgADH* under various environmental stresses was analyzed at different time points using real-time PCR. ABA, SA and especially JA (80-fold) significantly induced *PgADH* expression within 24 h of treatment. The positive responses of *PgADH* to abiotic stimuli suggest that ginseng ADH may protect against hormone-related environmental stresses. [BMB reports 2009; 42(10): 673-678]

### INTRODUCTION

Alcohol dehydrogenase (ADH; alcohol:NAD<sup>+</sup> oxidoreductase, EC 1.1.1.1) catalyzes the reversible conversion of aldehydes to corresponding alcohols. The enzyme is presumably required by plants for the reduction of acetaldehyde to ethanol during NADH metabolism in response to periods of anaerobic stress. As ADH activity is considered essential for the survival of plants during anaerobic conditions, much of the research on ADH in plants has been focused on its response to anaerobic stress (1).

In addition to hypoxia or anoxia, other environmental stresses such as cold and dehydration induce the expression of the ADH gene in *Arabidopsis thaliana* (2). ADHs are also implicated in the response to a wide range of other stresses, elicitors and ABA (3, 4). Some reports indicate that Ca<sup>2+</sup> may play

an important role in the induction of ADH gene expression during anoxia (5). Moreover, recent genetic evidence suggests that both an ABA-dependent and an ABA-independent pathway are involved in the ADH-mediated stress response (6).

ADHs are of multiple occurrences, separate origin and possess different activity patterns in nature. Medium-chain dehydrogenases/reductases (MDR) (7) and short-chain dehydrogenases/reductases (SDR) (8) are two large superfamilies, both of which include ADH enzymes. SDR is a very large enzyme family consisting mostly of NAD- or NADP-dependent oxidoreductases. The original name of this family was 'insect-type' or 'short-chain' alcohol dehydrogenases, as the first member to be characterized was *Drosophila* ADH (9). All these proteins consisted of polypeptides about 250 amino acid residues in length. Most members of this family are characterized by distant duplications and divergence, and lack zinc-liganding cysteine residues in their coenzyme binding regions (8-10). Most dehydrogenases possess at least 2 domains: the first binds the coenzyme while the second binds the substrate. This latter domain determines substrate specificity and contains amino acids involved in catalysis. Little sequence similarity has been found in the coenzyme-binding domain, and therefore it has been suggested that the structure of SDRs was produced from gene fusion of a common ancestral coenzyme nucleotide sequence with various substrate specific domains (11).

In contrast, most ADHs from other organisms, including those of mammals, plants and yeasts, belong to the MDRs, which have a longer polypeptide of about 370 residues and usually contain zinc ligands in their active site (10). Most plant ADHs are Zn-dependent MDRs. A few representative SDRs have been cloned from plant sources, but the molecular functions of only a few are known (12).

There are currently no reports on ADH in ginseng plant. *Panax ginseng* C. A. Meyer is a perennial herb of the family *Araliaceae* and is cultivated for its root that is highly valued for medicinal purposes. Similar to other crop plants, ginseng experiences various stresses during its life cycle. The cultivation of ginseng, specifically, requires a long period and is therefore more susceptible to abiotic stresses. However, despite the purported medicinal importance of ginseng, very little genomic research has been performed. In this study, we report the cloning of the SDR-ADH gene from *P. ginseng* and provide de-

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Received 8 April 2009, Accepted 30 May 2009

**Keywords:** Abiotic stress, Gene expression, Ginseng, JA, Short chain-alcohol dehydrogenase

tailed analyses of the expression profile of genes activated in response to abiotic stresses.

## RESULTS

### Isolation and amino acid sequence analysis of *PgADH*

Using a cDNA library prepared from the leaf of *P. ginseng*, we performed an expressed sequence tag (EST) analysis in which we identified a cDNA clone encoding an ADH gene. We named this gene *PgADH* (*P. ginseng* alcohol dehydrogenase) and deposited its sequence data in GenBank under accession number FJ518865. The *PgADH* cDNA was 1,106 nucleotides long and had a putative open reading frame of 801 bp. This ORF encodes an alcohol dehydrogenase protein of 266 amino acids with a predicted molecular weight of 29 kDa and isoelectric point of 6.84. The instability index (II) was computed to be 22.47, which classifies the protein as stable. The aliphatic index, regarded as a positive factor for increased thermostability, was calculated as 98.27 using ProtParam (13).

### Homology analysis

A GenBank BlastX search revealed that the deduced amino acid sequence of *PgADH* shares a high degree of homology with the SDR-ADH proteins of *Prunus armeniaca* (69% identity, 86% similarity), *Prunus mume* (68% identity, 85% similarity), *Cucumis melo* (68% identity, 85% similarity) and *Arabidopsis thaliana* (60% identity, 78% similarity). In contrast, *PgADH* shares lower than 10% identity with me-

dium-chain ADH proteins from other plants (data not shown). Alignment of the SDR-ADHs indicates that amino acid residues are highly conserved (Fig. 1).

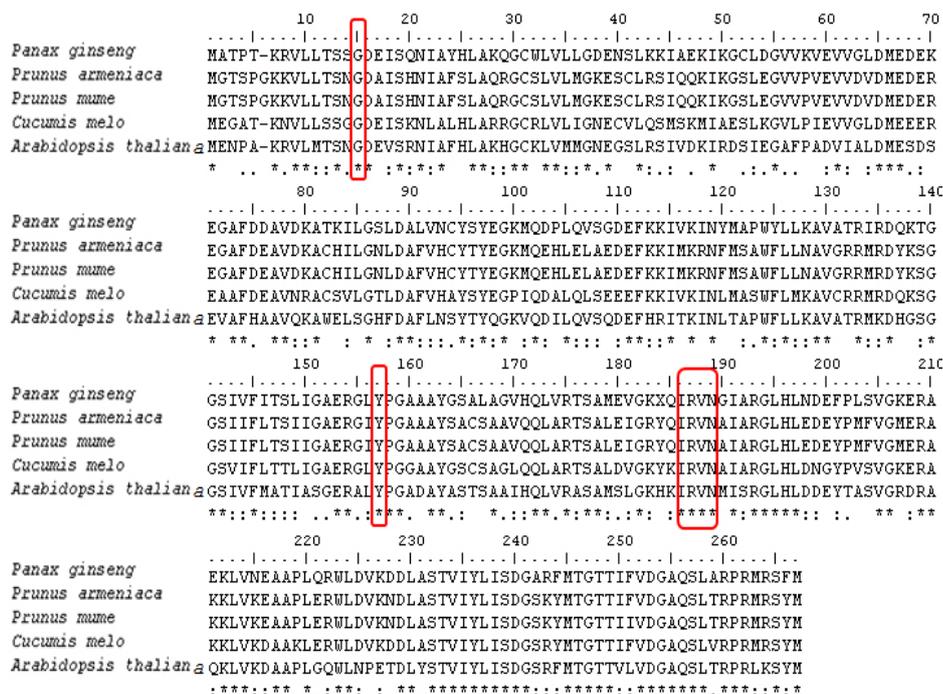
Secondary structure analysis and molecular modeling for *PgADH* were performed by SOMPA. The secondary structure analysis revealed that *PgADH* consists of 117 residues that form  $\alpha$ -helices, 23 residues that form  $\beta$ -turns jointed by 45 residue extended strands, and 81 residues that form random coils (Fig. 2A). This result is highly similar to the ADHs of *P. armeniaca*, whose secondary structure contains 125 residues that form  $\alpha$ -helices, 23 residues that form  $\beta$ -turns jointed by 45 residue extended strands, and 74 residues that form random coils. The hydrophilicity profiles of these SDR-ADHs also exhibited a similar pattern (Fig. 2B).

### DNA hybridization analysis of *PgADH*

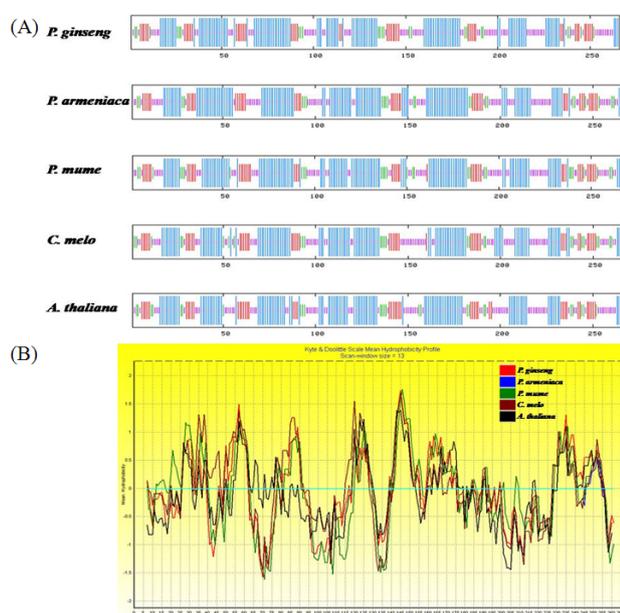
The copy number of the *PgADH* gene was examined using genomic DNA hybridization analysis. Fig. 3 shows the hybridization pattern of ginseng DNA digested with two different restriction enzymes. A single band was observed upon *Bam*HI digestion, whereas three hybridization bands were observed with *Hind*III digestion. This indicates that a multi-gene family encodes *PgADH* or related proteins.

### Temporal expression of the *PgADH* gene in response to abiotic stresses

The expression of *PgADH* at different time points after various treatments was analyzed by real-time PCR. Fig. 4A shows the



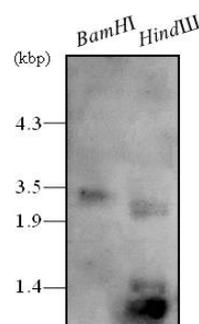
**Fig. 1.** Multiple alignment of the deduced amino acid sequences of *PgADH* with short-chain ADH from other plants; *Prunus armeniaca* (A BZ79222), *Prunus mume* (BAE4866 2), *Cucumis melo* (ABC02082) and *Arabidopsis thaliana* (AAM65725). \*, :, and . indicate conservative, identical, and weakly similar amino respectively. Box indicates conserved residues within the SDR family.



**Fig. 2.** Superimposed hydrophilicity profiles and secondary structure predictions for SDR-ADH. (A) Comparison of the secondary structures of *PgADH*, *P. armeniaca*, *P. mume* and *C. melo* by SOPMA. The helix, sheet, turn and coil are indicated in order from the longest to the shortest. (B) Hydropathic index analysis. Hydrophobic domains are indicated by positive numbers; hydrophilic domains are above line and hydrophilic domains are below.

expression pattern of *PgADH* in response to chilling stress. The level of *PgADH* mRNA declined rapidly within 1 h and continued to decrease until 4–8 h post-treatment, where gene expression was only 0.02-fold of control. Following this, the expression level then increased to 2.04-fold at 72 h post-treatment. Fig. 4B shows the expression of *PgADH* against anoxia stress. At 1 h post-treatment, gene expression was 0.33-fold of control and maintained lower level during the following 4–8 h. However, *PgADH* expression dramatically increased to 3.94-fold at 48 h, followed by another decreased at 72 h (0.81-fold). Fig. 4C shows the accumulation of *PgADH* mRNA in response to exogenous hydrogen peroxide. Expression increased to 2.03-fold at 1 h and then continued to increase until 48 h post-treatment. The *PgADH* transcript level was highest at 48 h (7.71-fold) and then decreased to 5.77-fold at 72 h. Upon cadmium treatment, the transcription of *PgADH* gradually decreased until 24 h post-treatment (0.14-fold), followed by an increase to maximum accumulation (3-fold) at 72 h (Fig. 4D). Sucrose treatment induced an increase in the *PgADH* transcript level to 2.09-fold at 4 h (Fig. 4E). The expression level then decreased to 1.23-fold at 8 h, but then increased again from 24 to 72 h post-treatment (3.33-fold).

Fig. 4F shows the expression pattern of *PgADH* after wounding stress treatment. The mRNA level slightly increased to 1.77-fold at 1 h, but was then maintained at steady-state levels



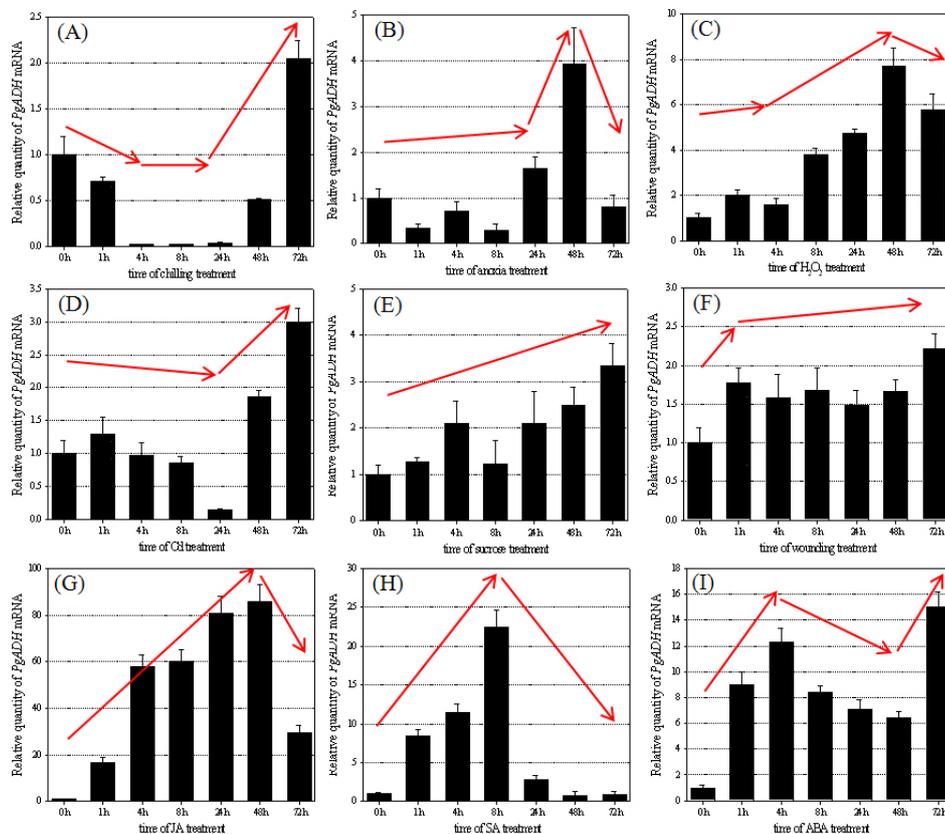
**Fig. 3.** Southern blot analysis of *PgADH*. Genomic DNA (10 µg per lane) isolated from *P. ginseng* leaves was digested overnight by *Bam*HI and *Hind*III, followed by hybridization with the coding sequence of *PgADH* as a probe.

until 72 h post-treatment (2.21-fold). As shown in Fig. 4G, a significant induction (16.68-fold) of *PgADH* expression was observed at 1 h post-treatment with jasmonic acid (JA). During JA stress, the level of *PgADH* mRNA was increased to within 57.72 to 80.81-fold within 4–24 h. The expression level continued to increase until 48 h post-treatment (85.59-fold), and then decreased to 29.66-fold at 72 h post-treatment. Salicylic acid (SA) treatment caused a rapid induction in *PgADH* expression within 1 h post-treatment (8.38-fold). Expression continued to increase to 11.4-fold at 4 h and peaked at 8 h (22.46-fold). However, *PgADH* expression decreased rapidly after the 24 h time point to 2.77-fold, and remained lower than the control level after 48–72 h (0.75- and 0.88-fold, respectively) (Fig. 4H). After exposure to abscisic acid (ABA) stress, *PgADH* mRNA increased to 8.96- to 12.29-fold within 1 to 4 h (Fig. 4I). Following this the expression level decreased steadily from 8 to 48 h post-treatment, yet remained 6.4-fold higher than control level. At 72 h expression was strongly induced to 15-fold.

## DISCUSSION

In the present study, we reported for the first time the isolation and characterization of the gene encoding ADH from ginseng. Searches of the BlastX database suggested that *PgADH* cDNA belongs to a SDR-ADH family of genes. Indeed, *PgADH* cDNA encodes an ADH protein of 266 amino acids and a predicted molecular weight of 29 kDa, which is typical of SDR-ADH (8). This molecular weight is the same as that reported for the SDR-ADH of *C. melo* (14), which coincidentally has high homology with *PgADH*.

Persson *et al.* (9) emphasized several conserved elements of unknown function in plant SDRs. Some of these conserved regions are present in *PgADH* and homologous SDR-ADH, which corresponds with the behavior of conserved residues in melon SDR-ADH (14). Gly 15 is conserved in *PgADH* (Fig. 1), which is consistent with reports on other plant SDR-ADHs



**Fig. 4.** Relative quantities of *PgADH* mRNA at various time points post-treatment with various stresses. (A) Chilling, (B) anoxia, (C) H<sub>2</sub>O<sub>2</sub>, (D) CdSO<sub>4</sub>, (E) sucrose, (F) wounding, (G) JA, (H) SA, (I) ABA. The error bars represent the standard error of the means of three independent replicates.

(14). Within certain SDR proteins, the section proximal to the variable substrate-binding loop contains a sequence of polar and hydrophobic I-R-V-N residues (positions 186-189). These residues are known to connect the substrate binding loop to the active site (15). In *Drosophila* ADH, the mid-chain contains a conserved, catalytically active triad of polar S, Y, K residues of which Tyr is the most conserved out of the whole family (9). This could explain why only Tyr 157 is observed in our results out of all triad residues. All previous studies demonstrate that Tyr functions acts as a catalytic base (15). The lack of other known conserved residues in SDRs can be justified by their numerous biological functions as well as by their wide range of substrates in higher plants (14). Despite the variability of SDR-ADHs, some important residues are conserved in *PgADH*. Indeed, alignment of *PgADH* with other plant SDR-ADHs reveals a number of undemonstrated conserved residues. In addition, analysis revealed that the secondary structure of *PgADH* is highly similar to that of SDR-ADHs, and that a high similarity in hydrophilicity pattern also exists (Fig. 2). These results indicate that *PgADH* and other plant SDR-ADHs have evolved from SDR as evidenced by their roles.

According to Gottlieb (16), most plants have two or three ADH isozymes existing as both hetero- and homodimers in various organs. Correspondingly, multiple hybridization bands

were observed in the genomic DNA blot of *PgADH*, indicating that *PgADH* is present as a multi-gene family comprised of similar or related genes throughout the ginseng genome. This observation is in agreement with southern blot analysis of pea SDR-ADH, which was confirmed to consist of at least three genes (17).

Plant ADHs are implicated in the response to adverse environmental stresses. When ginseng plantlets were exposed to stresses, *PgADH* was differentially expressed depending on the exposure time. In ginseng, *PgADH* expression seems to be down-regulated by chilling at an early stage, but is then increased by 2-fold at 72 h post-treatment. Chilling is known to trigger oxidative stress, which correspondingly, strongly up-regulates *PgADH* expression when mediated by H<sub>2</sub>O<sub>2</sub> treatment. Cold stress has been reported to increase ADH in other plants such as rice (4), and *Arabidopsis* (2). One hypothesis is that cold stress may disrupt respiration in plants, which forces some plants to compensate for impaired mitochondrial function by shifting their metabolism from aerobic to anaerobic respiration (4). In potato, a wide range of stresses, including treatment with SA, and anaerobiosis, was shown to induce accumulation of ADH expression (3). Similarly, our results also show that *PgADH* is induced by anoxia and SA (Fig. 4). Heavy metal treatment, which also leads to oxidative stress in plants,

induces *PgADH* expression by 3-fold. The mRNA level of pea ADH is also transiently increased, returning to control levels upon exposure to heavy metal stress (17). The expression of *PgADH* upon sucrose treatment is increased by 3.5-fold at 72 h post-treatment. Changes in the activity of grapevine ADH were observed in relation to sugar content, suggesting a link between increase in ADH activity and the activities of enzymes related to sucrose catabolism (18).

In the present study, we demonstrated that *PgADH* was regulated by the signaling molecules SA, JA and ABA on the transcriptional level. The exogenous application of these hormones triggered an early and significant induction of *PgADH*. Wounding as well as SA and JA treatment impact *PgADH* expression, which corroborates a report by Brosche and Strid (17) that found pea SDR-ADH could be a component of systemic acquired resistance. In Arabidopsis, ADH mRNA levels were also increased upon ABA treatment (19). It is well known that ABA modulates gene expression under environmental stresses such as salinity, cold acclimation and wounding (20, 21).

There are few reports of the molecular characterization of SDR-ADH in plants. Our results confirm that *PgADH* is associated with environmental stresses. Information on SDR-ADHs in plants is scarce, therefore this study may provide evidence justifying the further characterization of SDR-ADHs. Our results show that *PgADH* expression was increased but not significantly up-regulated by oxidative stresses such as chilling, as well as by heavy metals, anoxia, wounding and osmotic stress. Such stresses seem to weakly influence *PgADH* at an early stage, but eventually lead to elevated levels of *PgADH* later on after their administration. It was noticeable that H<sub>2</sub>O<sub>2</sub> and plant hormones induce strong accumulation of *PgADH* expression, possibly by triggering a signal transduction cascade. Both H<sub>2</sub>O<sub>2</sub> and hormones seem to be unrelated, but recent studies reveal that H<sub>2</sub>O<sub>2</sub> is related with JA in wound-induced defense response (22). As JA had the strongest impact on *PgADH* expression, *PgADH* could be related with wounding, a common inducer of hormones and H<sub>2</sub>O<sub>2</sub>. Further research needs to examine this possibility. It is certain that *PgADH* plays a specific role related with hormones and is involved in plant defense against environmental stresses.

## MATERIALS AND METHODS

### Nucleotide sequencing and sequence analysis from cDNA library

Total RNA was isolated from leaves of a 4-year-old ginseng plant followed by construction of a cDNA library as previously described (23). Homologous sequences of ADH EST were searched for using the GenBank databases and a BLASTX algorithm. These deduced amino acid sequences were then used to search for homologous proteins in the databases using BLAST network services at NCBI. We used ClustalX with default gap penalties to perform a multiple alignment of ADH isolated in ginseng and other previously registered species.

The protein properties were estimated using ProtParam (13) and the hydrophathy value was calculated by the Kyte and Doolittle method (24).

### Southern blot analysis

Genomic DNA was extracted from ginseng leaf tissue using a DNA mini kit (GENEAll Biotechnology Inc, South Korea). Approximately 10 µg of DNA were digested with *Bam*HI or *Hind*III, and the digested samples were subjected to electrophoresis on a 1.3% agarose gel. The separated DNA fragments were transferred to a nylon membrane using a Turbo blotter apparatus (Schleicher and Schuell, UK) in 10X SSC. The DNA fragments were cross-linked to the membrane by UV-irradiation and then were pre-hybridized with DIG Easy Hyb Solution (0.1 ml/cm<sup>2</sup> of membrane; Roche Applied Science, Indianapolis, IN) containing 100 µg of fragmented ginseng DNA for 3 h at 37°C. Probe labeling, hybridization and detection was performed using DIG High Prime DNA Labeling and Detection Starter Kit according to manufacturer's recommended protocols with the same primers as RT-PCR.

### Plant materials and application of environmental stresses

*P. ginseng* cv. "Hwang-Sook" seeds (provided by Ginseng Genetic Resource Bank) were cultured into 3-week-old plantlets that were used for the treatments and nucleic acid extractions, as previously described (23). For chemical stress or plant hormone treatments, the plantlets were placed for various periods in Murashige and Skoog (MS) medium containing the indicated concentrations of chemicals; 10 mM H<sub>2</sub>O<sub>2</sub>, 20% sucrose, 500 µM CdSO<sub>4</sub>, or 1 mM SA, 0.2 mM JA, 0.1 mM ABA. Chilling stress was applied by exposing the plantlets at 4°C. Anoxic conditions were generated by submerging whole potted seedlings in water up to the apex of the root for the duration of the experiment. For mechanical wounding stress, the leaves and stems of seedlings were wounded with a scalpel. In all cases, stress treatments of the 10 plantlets were performed on MS media for 1, 4, 8, 24, 48 or 72 hours. Control plants were held in a growth room at 25°C under a 16 h photoperiod. Upon completion of treatments, stressed plant materials were immediately frozen in liquid nitrogen and stored at -70°C until required.

### Real time quantitative RT-PCR

Total RNA was extracted from seedlings of *P. ginseng* using an RNeasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR, 200 ng of total RNA served as a template for reverse transcription using a Power cDNA synthesis kit (INTRON Biotechnology, Inc., South Korea). Real-time quantitative PCR was performed using 100 ng of cDNA in a 10-µl reaction volume using SYBR<sup>®</sup> Green SensimixPlus Master Mix (Quanta, Watford, England). Primers specific for *PgADH*, 5'-AGTTAGG ACATCCGCAATGG-3' and 5'-GAAGCGTGCACCATCACTTA-3' were used in real time-PCR. The thermal cycler conditions recommended by the manufacturer were used as follows: 10

min at 95°C, followed 40 cycles of 95°C for 10 s, 58°C for 10 s and 72°C 20 s. The fluorescent product was detected at the last step of each cycle. Amplification, detection and data analysis were performed using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). Threshold cycle (Ct) represents the number of cycles at which the fluorescence intensity was significantly higher than the background fluorescence at the initial exponential phase of PCR amplification. To determine the relative fold differences in template abundance for each sample, the Ct value for *PgADH* was normalized to the Ct value for  $\beta$ -actin and was calculated relative to a calibrator using the formula  $2^{-\Delta\Delta Ct}$ . Three independent experiments were performed.

### Acknowledgements

This study was supported by KGCMVP for Technology Development Program of Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

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