Isolation of S-adenosyl-L-methionine synthetase gene from *Panax ginseng* C.A. Meyer and analysis of its response to abiotic stresses

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ABSTRACT

A cDNA clone containing a S-adenosyl-L-methionine synthetase (SAMS) gene, named as *PgSAM*, was isolated from a commercial medicinal plant *Panax ginseng*. *PgSAM* is predicted to encode a precursor protein of 307 amino acid residues, and its sequence shares high homology with a number of other plant SAMS. *PgSAM* is expressed at different levels in various organs of ginseng. The expression of *PgSAM* in adventitious roots and hairy roots of *P. ginseng* were analyzed using reverse transcriptase (RT)-PCR and real-time PCR under various abiotic stresses. Salt, salicylic acid, abscisic acid and chilling stresses induced *PgSAM* significantly at different time points within 2–72 h post-treatment. This study revealed that *PgSAM* may help to protect the plants against various abiotic stresses. [Physiol. Mol. Biol. Plants 2009; 15(3) : 267–275] E-mail : deokchunyang@yahoo.co.kr

Key words : Abiotic stress, *Panax ginseng*, S-Adenosyl-L-Methionine Synthetase (SAMS), Abscisic acid, Salicylic acid, Semi-quantitative RT-PCR, real-time PCR

INTRODUCTION

S-adenosyl-L-methionine synthetase (SAMS, E.C. 2.5.1.6) is a ubiquitous enzyme found in all living organisms. This synthetase catalyzes the formation of S-adenosyl methionine (SAM) from L-methionine and adenosine triphosphate (ATP) (Boerjan *et al.*, 1994). SAM is the major methyl-group donor for numerous transmethylation reactions in both prokaryotes and eukaryotes (Gonzalez *et al.*, 2000). SAM is required for the biosynthesis of phenylpropanoid compounds (Campbell and Sederoff, 1996) and is also a precursor for the biosynthesis of polyamines and the phytohormone ethylene (Tiburcio *et al.*, 1990; Kende, 1993). Decarboxylated S-adenosyl methionine serves also as an aminopropyl group donor, which contributes to the formation of polyamine, spermidine and spermine that are involved in cellular

multiplication and differentiation, macromolecular synthesis, and membrane stabilization (Cazzulo, 2003). Moreover, SAM has recently been shown to be a potential source of 5P-deoxyadenosyl radicals in a large number of reductive metabolic processes (Fontecave *et al.*, 2001).

SAM synthase (SAMS) has been cloned from several plant species, and small gene families were discovered in all cases (Espartero *et al.*, 1994; Van Breusegem *et al.*, 1994; Peleman *et al.*, 1989; Schroder *et al.*, 1997; Shen and Tarczynski 2002). The genome of *Arabidopsis thaliana* has four genes encoding putative SAMS (The Arabidopsis Information Resource, <http://www.arabidopsis.org/>). *SAM1* and *SAM2*, are the two of these genes, which are 97% identical. These genes expressed highly in stems and roots (Peleman *et al.*, 1989). Moreover, a GUS reporter fused to the 5'-UTR of *SAM1* was preferentially expressed in vascular tissues (Peleman *et al.*, 1989). *Lycopersicon esculentum* (tomato) and *Catharanthus roseus* (vinca rosea) possess at least

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three and four SAMS genes, respectively, that are differentially transcribed in response to various stress conditions including salinity (Van Breusegem *et al.*, 1994; Schroder *et al.*, 1997). In tomato, mRNAs of *SAM1* and *SAM3* were specifically accumulated in response to NaCl, mannitol or abscisic acid (ABA) treatments (Van Breusegem *et al.*, 1994). In *C. roseus* seedlings, NaCl treatment led to a significant increase of transcripts from all SAMS isogenes (Schroder *et al.*, 1997). In rice, the transcript of single SAMS gene was also accumulated under water stress (Van Breusegem *et al.*, 1994).

This is the first report on isolation of an *S*-adenosyl-L-methionine synthetase gene from an oriental medicinal plant, *Panax ginseng* C.A. Meyer, that is one of the most valued medicinal plants widely used in Asian countries. In spite of its economic and medicinal importance, little studies were done about ginseng genomics. In this study, an *S*-adenosyl-L-methionine synthetase (*PgSAM*) gene was isolated and its expression profiles in response to various abiotic stresses were studied.

MATERIALS AND METHODS

Plant Materials

Panax ginseng adventitious roots and hairy roots were collected from Korean Ginseng Center and Ginseng Genetic Resource Bank, Kyung Hee University and cultured in Gamborg's medium (B5 medium) supplemented with 3 % sucrose and 2 mg/L Indole Butyric Acid (IBA) and Murashige & Skoog (MS) suspension medium without any hormone respectively. Both the roots were maintained by regular subculture in every 4 weeks. Abiotic stress treatment was carried out with one month- subculture roots.

RNA isolation and construction of a cDNA library

Total RNA was isolated from roots of a 4-year-old ginseng using the aqueous phenol extraction procedure as described by Morris *et al.* (1990). The poly (A)⁺ RNA was isolated via oligo-dT-cellulose chromatography using an mRNA isolation kit (Stratagene, <http://www.stratagene.com>). A commercial cDNA synthesis kit (TriplEx2, Clontech) and a GigapackIII Gold packaging extract (Stratagene, <http://www.stratagene.com>) were utilized in the construction of the cDNA library, in accordance with the instruction manual provided by the manufacturer (Clontech, <http://www.clontech.com>). The

fractions containing cDNAs larger than 500 bp were recovered, and used for cDNA library construction.

Nucleotide sequencing and sequence analysis

pTriplEx phagemids were excised from the λ pTriplEx2 and used as templates for sequence analysis. The 5' ends of cDNA inserts was sequenced by an automatic DNA sequencer (ABI prism 3700 DNA sequencer, Perkin-Elmer, USA). Homologous sequences of SAM EST were searched against the GenBank databases using a BLASTX algorithm. A pTriplEx phagemid for SAM cDNA was excised from the λ pTriplEx2 and used as template for sequence analysis. Nucleotide and amino acid sequence analyses were performed using DNASIS program (Hitachi).

These deduced amino acid sequences were searched for homologous proteins in the databases using BLAST network services at the National Center for Biotechnology Information. We used ClustalX with default gap penalties to perform multiple alignment of SAM isolated in ginseng and previously registered in other species. Based on this alignment, a phylogenetic tree was constructed according to the neighbour joining method using the MEGA3 programs. The protein properties are estimated using ProtParam (Gasteiger *et al.*, 2005) and the hydropathy value is calculated by the method of Kyte and Doolittle (1982).

DNA hybridization analysis

Korean ginseng adventitious root DNA (approximately 20 μ g) was digested with EcoRI and XbaI, the digested samples were subjected to electrophoresis on a 0.8 % agarose gel. The separated DNA fragments were transferred to a Zeta-probe-GT nylon membrane (BioRad, Hercules, CA) by downward capillary alkaline transfer. The DNA fragments were cross-linked to the membrane by UV-irradiation and pre-hybridized with DIG Easy Hyb solution (0.1 mL/cm² of membrane; Roche Applied Science, Indianapolis, IN) containing 200 μ g of fragmented salmon sperm DNA for 1 h at 39 °C. Hybridization with a DIG-labeled probe was carried out at 39 °C overnight. The blot was washed twice with low stringency wash buffer (2 $\times$ SSC, 0.1 % SDS) for 5 min each at room temperature, and twice with high stringency buffer (0.1 $\times$ SSC, 0.1 % SDS) for 30 min each at 65 °C. The *PgSAM* was detected by using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science) following the manufacturer's protocol. The DNA probe was labeled with digoxigenin-11-dUTP by random priming, using the DIG High Prime DNA Labeling and Detection Starter Kit I according to the

manufacturer's recommendations. The DNA probe was synthesized using PCR with primer on the 3' untranslated region of *PgSAM*.

Stress treatment

To investigate the response of *PgSAM* gene to various stresses, one month subculture adventitious roots and hairy roots were used. Chilling stress was applied by exposing the adventitious roots and hairy roots separately to 4 °C and root samples were treated with 100 mM salt (NaCl), and 10 mM salicylic acid (SA). In addition to this, adventitious roots alone treated with 100 mM abscisic acid (ABA) and without any reagent separately. Each treatment sample was collected at time points 0, 2, 4, 8, 12, 24 and 48 hrs respectively. Hairy root samples were collected at 72 hrs also. All the treated plant materials were immediately frozen in liquid nitrogen and stored at -70 °C until required.

3' cDNA end amplification of *PgSAM*

The first strand cDNA was synthesized from 3 µg of total RNA according to the protocol of 3'RACE system for the CapFishing Full-length cDNA Premix Kit (Seegene, Korea) using the Oligo dT-adaptor primer (5'-CTG TGA ATG CTG CGA CTA CGA T(T)₁₈-3') provided by Seegene (Korea). For the amplification of 3' end of *PgSAM*, a gene specific primer 5'-GSP (5'-TCC TTG TTA ACA TTG AGC AGC AGA GTC-3') was designed and synthesized as per the conserved region known sequence from ESTs of *Panax ginseng*. PCR reaction volume contained 5 µl cDNA, 10 pmol of 5'GSP, 10 pmol of 3'RACE, 10 µl 2X SeeAmp™ Taq Plus Master Mix and finally adjusted to 20 µl reaction volume. PCR was performed using following protocol: the template was denatured at 94 °C for 3 min followed by 30 cycles of amplification (94 °C for 40 sec, 64 °C for 40 sec, 72 °C for 60 sec) and extension by 5 min at 72 °C.

5' cDNA end amplification of *PgSAM*

The 5' end of the cDNA sequence was obtained according to the protocol of the 5'RACE system for the CapFishing Full-length cDNA Premix Kit (Seegene, Korea). Complementary reverse gene specific primers 3'GSP (5'-GAT GGG TTA AGG TGG AAA ATG GTC TTT-3') was designed and synthesized based on the EST sequences and 5'RACE-primer (5'-GTC TAC CAG GCA TTC GCT TCA T-3') as the forward primer. PCR was carried out by denaturing cDNA at 94 °C for 3 min followed by 30 cycles of amplification (94 °C for 40 sec, 64 °C for 40 sec, 72 °C for 90 sec) and by 5 min at 72 °C.

Sequencing of the PCR products

The amplified fragments of interest were excised from 2 % agarose gel and purified with the Agarose Gel DNA extraction kit (GeneAll, Korea). The fragments were then cloned into the pGEM-T Easy Vector System (Promega, USA), transformed into *E. coli* XL1-Blue and plasmids were purified with Plasmid SV mini (GeneAll, Korea). Sequencing reactions were carried out using Big Dye (Genotech, Korea) according to the manufacturer's protocol, and the sequences were obtained using an ABI 3700 Genetic Analyzer (Genotech, Korea).

Full-length *PgSAM* cDNA amplification

The sequences of 3' and 5' cDNA end products were assembled to generate a putative full-length cDNA of *PgSAM*. A pair of PCR primers, SAM-F (5'-AAGAAATGGAGACCTTTCTAT3') and SAM-R(5'-AAACTGACAAAGGCTCAGGAAC-3'), were designed for the amplification of full-length cDNA according to the assembled sequence of *PgSAM*.

Real time quantitative RT-PCR

Total RNA was extracted from hairy roots of *P. ginseng* using RNeasy mini kit (Qiagen, Valencia, CA, USA). For quantitative RT-PCR, 200 ng of total RNA was used as a template for reverse transcription using oligo(dT)15 primer (0.2 mM) and AMV Reverse Transcriptase (10 U/µl) (INTRON Biotechnology, Inc., South Korea) according to the manufacturer's instructions. Real-time quantitative PCR was performed using 100 ng of cDNA in a 10-µl reaction volume using SYBR®Green SensimixPlus Master Mix (Quantace, Watford, England). Specific primers for *PgSAM*, 5'-AGC AGC AGA GTC CTG ATA TTG C-3' and 5'-ACA AGT ACC ATT CTT GCG GAC T-3' were used for perform real time-PCR. The thermal cycler conditions recommended by the manufacturer were used as follow: 10 min at 95 °C, followed 40 cycles of 95 °C for 10 s, 60 °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 carried out with 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 *PgSAM* was normalized to the Ct value for β-actin (5'-GAT GAC ATG GAA AAG ATT TGG CAT C-3') (5'-TGT TGT ACG ACC

ACT AGC ATA CAG G-3') and calculated relative to a calibrator using the formula $2^{-\Delta\Delta C_t}$. Three independent experiments were performed.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from stress treated adventitious roots of *P. ginseng* using RNeasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR, 2 µg of total RNA was used as a template for reverse transcription. Oligo (dT)₁₅ primer (0.2mM) (INTRON Biotechnology,

Inc., South Korea) was added and the mixture was heated for 5 min at 75 °C. Then reaction mixture was incubated with AMV Reverse Transcriptase (10 U/µl) (INTRON Biotechnology, Inc., South Korea) for 60 min at 42 °C. The reaction was inactivated by heating the mixture at 94 °C for 5 min. PCR was then performed using a 1 µl aliquot of the first stand cDNA in a final volume of 25 µl containing 10 pmol of specific primers for coding of *PgSAM* gene (forward, 5'-CAA GCT TGG TTG CAA GAA CA-3'; (reverse) 5'-ATG AGG GAC ACC CTG ACA AG-

Fig. 1. Alignment of *PgSAM* with most closely related SAMS from Lychee (*Litchi chinensis*, AP13994), Papaya (*Carica papaya*, AAN07179), Vinca rosea (*Catharanthus roseus*, Q96551), beetroot (*Beta vulgaris*, BAE07180) and rice (*Oryza officinalis*, CAJ45561). Gaps are marked with dashes. Amino acid with in box represents conserved motif SAM 1 and Box with underlined sequence represents conserved motif SAM 2 segments respectively.

3' were used. As a control, the primers specific to *P. ginseng* actin gene were used. (forward, 5'- CGT GAT CTT ACA GAT AGC TTG ATG-3'; reverse, 5'- AGA GAA GCT AAG ATT GAT CCT CC-3').

RESULTS AND DISCUSSION

Isolation and Characterization of a cDNA encoding *PgSAM* Gene

Ginseng is very important medicinal plant. Its root has been used since ancient times as herbal medicine that provides resistance to stress, disease, and exhaustion (Jung *et al.*, 2003). As part of a genomic project to identify genes of the medicinal plant *P. ginseng*, a cDNA library consisting about 20,000 cDNAs were previously constructed. A cDNA encoding S-adenosyl-L-methionine synthetase (SAMS), designated *PgSAM*, was isolated and sequenced. The sequence of *PgSAM* has been deposited in GenBank under accession number EU703623.

PgSAM encodes a precursor protein of 307 amino acids. The calculated molecular mass of the mature protein is approximately 33.03 kDa with a predicted isoelectric point of 5.28. In the deduced amino acid sequence of *PgSAM* protein, the total number of negatively charged residues (Asp + Glu) was 41 while the total number of positively charged residues (Arg + Lys) was 29 (ProtParam). In addition, the amino acid sequence contains the two conserved SAMS motifs that are present in all plant SAMS (Horikawa *et al.*, 1990; Bairoch, 1993). The first motif is a hexapeptide (GAGDQG) that is thought to bind to the adenine moiety of ATP (Pajares *et al.*, 1991). The other motif is a glycine-rich nonapeptide (GGGAFSGKD) constituting a P-loop-like sequence, possibly involved in binding ATP (Takusagawa *et al.*, 1996) (Fig. 1). Neighbor-joining method (Saitou and Nei, 1987) was used for construction of phylogenetic tree based on SAMS amino acid sequences to show relationships between *PgSAM* and other plant SAMS. Phylogenetic analysis of plant SAMS has been carried out using the Clustal X program (Fig.2).

Fig. 2. Phylogenetic tree based on amino acid sequence, showing the Phylogenetic relation between *PgSAM* and other plant SAMS. The tree was constructed using Clustal X (Neighbor-joining method) and a bar represents 0.01 substitutions per amino acid position.

Fig. 3. Southern blot analysis of genomic DNA extracted from *P. ginseng* adventitious root equal amounts (10 μ g) of genomic DNA were digested with restriction enzymes, separated by electrophoresis, and transferred onto a Zeta-probe GT membrane. The blot was hybridized with the 32 P-labeled *PgSAM*-specific DNA fragments. Size markers are shown on the left.

DNA hybridization analysis of *PgSAM* Gene

The copy number of *PgSAM* gene was examined using genomic DNA hybridization analysis. (Fig. 3) shows the hybridization patterns of ginseng DNA digested with two different restriction enzymes. Multiple hybridization bands were observed with each digest, indicating that a multi-gene family encodes *PgSAM* or related enzymes.

The Differential Expression of *PgSAM* in Different *P. ginseng* Organs

The expression patterns of *PgSAM* in different *P. ginseng* organs were examined using quantitative real-time PCR. From the results shown in (Fig. 4A), it is clear that *PgSAM* is constitutively expressed in roots, leaves, shoot and adventitious roots. Among these organs, relatively higher levels of *PgSAM* transcripts (66-folds) were observed in roots. A moderate level of *PgSAM* was observed in adventitious roots where as in hairy root samples week expression was noticed. Peleman (1989) noticed that *SAM* genes expressed highly in stems and roots.

Fig. 4. Relative quantities of *PgSAM* mRNA in various organs of ginseng plant and analyses of transcripts in hairy roots of *P. ginseng* at various time points (Hrs) post-treatment with various stresses. A, various organs of ginseng where R, root, L, leaf, St, shoot, Ad, adventitious roots and Hr, hairy roots; B, 100 mM NaCl; C, 10 mM SA and D, Chilling (4 °C).

Differential expressions of the *PgSAM* under various abiotic stresses

The expression patterns of the *PgSAM* under various stresses, such as stress-related chemicals, NaCl (100 mM), SA (10 mM), ABA (100 mM) and Chilling stress samples were investigated by RT-PCR and real-time PCR.

First, *PgSAM* expression was investigated in adventitious roots culture in normal MS medium as a standard. Expression pattern was same at all the time points (Fig. 5A). Under salt stress, *PgSAM* transcripts were increased by 41-fold at 12 hrs post treatment and then decreased in hairy root samples (Fig. 4B). In the same way expression of *PgSAM* in adventitious roots was induced at 2 hrs, gradually increased and suddenly decreased at 48 hrs post treatment (Fig. 5B). Salinity affects photosynthesis in plants (Sudhir *et al.*, 2005). Osmotic potential increase during salt treatment could result from Na⁺ or Cl⁻ absorption and from the synthesis of compatible compounds (Pulla *et al.*, 2007; Caruso *et al.*, 2002). Plants respond to unfavorable environmental condition (i.e., alteration of the water status) accumulating polyamines (PAs) (Flores, 1991; Grillo and Colombatto, 2008; Groppa and Benavides, 2008) PAs most likely rigidify microsomal membrane surfaces, stabilizing them against NaCl and osmotic stress damage. Moreover, the higher levels of PAs bound to microsomal membranes most likely minimize the damaging effect of NaCl (Groppa and Benavides, 2008). SAM also functions as a precursor in the biosynthesis of the phytohormone ethylene and serves after decarboxylation as a propylamine group donor in the biosynthesis of polyamines (Van Breusegem *et al.*, 1994). This may be a reason for the induction of *PgSAM* to salt stress and its transcript levels increased gradually 2 hrs post treatment. Sanchez *et al.*, (2004) found tomato *SAM1* gene transcript was up-regulated in response to salt. Schroder *et al.*, (1997) also explained that exposure of the *C. roseus* seedlings to 25 or 85mM NaCl led to a significant increase of all transcripts (SAMS1, SAMS2 and SAMS3) in radicles and hypocotyls.

In salicylic acid treated hairy roots 20-fold transcripts were identified at 8 hrs post treatment (Fig. 4C). Similarly, expression pattern of *PgSAM* was induced strongly at 2 hrs, reached maximum at 8hrs and decreased 24 hrs post treatment in adventitious roots (Fig. 5C). Recently, Shen *et al.* (2002) reported that the *Arabidopsis* mto3 mutant, which lacks a functional SAM3 protein, shows a concomitant decrease in SAM levels and lignin content, these findings strongly suggest that a substantial amount of SAM protein activity in plants is

Fig. 5. RT-PCR analyses of the expressions of *PgSAM* gene in adventitious roots of *P. ginseng* at various time points (Hrs). with various stresses. A, without any reagent; B, 100 mM NaCl; C, 10 mM SA; D, 100 mM ABA and E, Chilling (4°C). Actin was used as an internal control.

committed to lignin biosynthesis. Lignin imparts strength to cell walls and impedes the degradation of wall polysaccharides thus acting as a major line of defense against pathogens, insects and other herbivores (Higuchi, 1981; Groppa and Benavides, 2008). SAM is a precursor for the biosynthesis of polyamines and the phytohormone ethylene (Tiburcio *et al.*, 1990; Kende, 1993). Ethylene has been shown to induce genes involved in pathogen defence (Boller, 1991). Van Breusegem (1994) reported that in parsley and alfalfa cells, *SAMS* gene expression was induced by exposure to a fungal elicitor (Kawalleck *et al.*, 1992) and yeast cell walls, respectively (Gowri *et al.*, 1991).

In case of chilling stress (4 °C) in hairy root samples 38-fold transcripts were observed at 48 hrs post treatment (Fig. 4D) where as in adventitious roots, *PgSAM* expression induced at 2 hrs and increased by time (Fig. 5D). Moreover, In ABA treatment expression pattern of *PgSAM* was induced at 2hrs then gradually increased until 48hrs post treatment (Fig. 5E). Many stress responses are regulated by ABA. ABA accumulation occurs in response to various stresses

that cause a decrease in tissue water content, such as dehydration induced by low water potential and salinity (Xiong and Zhu, 2003). Van Breusegem (1994) reported drought stress elevates rice *SAMS* mRNA levels 2- to 4-fold. Expression levels of *PgSAM* to all the stress reagents reveals that *SAMS* gene may play protective role in plants against abiotic stress.

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