Characterization of S-adenosylmethionine Synthetase Gene from Red Algae, Pyropia tenera

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ABSTRACT  S-adenosylmethionine (SAM) is widely involved in variety of biosynthetic processes. It participates in the methionine (Met) metabolic pathway. SAM synthetase (EC 2.5.1.6) is the main enzyme that creates SAM from Met and adenosine triphosphate. SAMS genes are highly conserved in different species. We have isolated SAMS genes from red algae, Pyropia tenera. It has been reported that SAMS genes can confer abiotic stress-related response and growth of plant organs in other species. To elucidate the function of PtSAMS gene in evolutionary perspective, we analyzed SAMS gene using bioinformatics tools and plant transformation of Arabidopsis.

Keywords  S-adenosylmethionine synthetase, SAMS, Pyropia tenera, Abiotic stress, Organ development

INTRODUCTION  S-adenosylmethionine synthetase (SAMS), also known as methionine adenosyltransferase (MAT), is an enzyme that synthesizes S-adenosylmethionine (AdoMet or SAM) from methionine and adenosine triphosphate (ATP) (Binet et al. 2011). SAM has been used in many cellular biochemistry reactions. It is the major hub of methionine metabolism (Martínez-López et al. 2008). SAM is a universal methyl donor. It plays a central role in most methylation of DNA, RNA, proteins, lipids, and polysaccharides in numerous enzyme-catalyzed reactions (Hilti et al. 2000).

SAMS gene in plant functions in organ development and stress response (Roeder et al. 2009). MAT3 is one of the 4 S-adenosyl methionine synthetase genes in Arabidopsis and is required pollen germination and pollen tube growth (Chen et al. 2016). PcSAMS1 and PcSAMS2 have been isolated from Pinus contorta (Lindroth et al. 2001). PcSAMS1 is expressed in roots and exhibits a specific expression pattern in the root meristem, whereas PcSAMS2 is expressed in roots as well as in shoots. SAMS have been reported from many plants, including Arabidopsis thaliana (Lindermayr et al. 2005; Mao et al. 2015), Oriza sativa (Van Breusegem et al. 1994), Pinus contorta (Lindroth et al. 2001), and Glycine max (Oh and Komatsu 2015). Differential expression patterns of SAMS have been found in different tissues of Arabidopsis (Shen et al. 2002; Mao et al. 2015). Functional studies have shown the effects of SAMS on abiotic stress. For example, overexpression of SAMS in alfalfa and tobacco could improve their salinity tolerance (Qi et al. 2010; Hua et al. 2012). Low temperature conditions can induce the expression of SAMS in O. sativa, A. thaliana, and Zea mays (Cui et al. 2005;
Consequently, it is important to determine the functions of SAMS in plant development and abiotic stress related mechanism. Stress-related gene SAMS (PySAMS) has been cloned from seaweed, Porphyra yezoensis (Yi et al. 2009). However, the exact functions of SAMS remain unknown. Therefore, the objective of this study was to perform functional analysis for PtSAMS through in silico study and generation of PtSAMS transgenic plant.

MATERIALS AND METHODS

Plant material and growth condition

Wild type seeds were surface sterilized in 70% ethanol containing 3.5% (w/v) household bleach and 0.1% (w/v) Tween 20 detergent for 20 min and subsequently rinsed by 100% ethanol 5 times (Kalifa et al. 2004). After entirely dried, sterilized seeds were carefully scattered onto 0.5 × Murashige and Skoog (MS) plate containing 0.8% agar and 2% sucrose. To improve the rate and synchrony of germination, plates were placed at 4°C for 3 days (Li et al. 2013). Arabidopsis were then grown at 22°C with light intensity of 120 μmol photons m⁻² sec⁻¹, 16/8 (light/dark) photoperiod, and relative humidity of 60%. After germination for 10 days, Arabidopsis seedlings were then transferred to soil. Arabidopsis were allowed to grow until bolting occurred on soil.

Construction of 35S::PtSAMS expression vector

The full-length cDNA of PtSAMS gene was amplified from Pyropia tenera cDNA library using PCR from Pyropia yezoensis sequence (GenBank: FJ404748.1). The following primers were used: PySAMS-OX-XbaI-F (5’-AATCTAGAATGGCAGCCATGAAGAAC-3’) and PySAMS-OX-SmaI-R (5’-AACCCGGGCTAGAAGCTCA AGC-3’). Underlines are restriction enzyme sites for XbaI and SmaI, respectively.

Generation of transgenic plants

To generated transgenic plants, Arabidopsis were transformed using GV3101 strain of Agrobacterium tumefaciens according to the floral dip protocol (Clough and Bent 1998). Transformed seeds (T0) were selected on 0.5×MS containing 4 mg/L phosphinothricin (PPT) and 250 mg/L cefotaxime.

Genomic DNA extraction and genotyping

Genomic DNAs of transgenic plants were extracted using GeneAll Plant SV kit (GeneAll, Seoul, Korea). bar gene selection of transgenic plants was carried out using PCR with the following primers: bar-F (5’-GGATCT-ACCATGAGCCAGA-3’) and bar-R (5’-GGAAAGTTGACCGTGCTTGTC-3’). PCR conditions included an initial denaturation for 94°C for 5 minutes followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, extension for 30 seconds at 72°C, and 7 minutes of final extension at 72°C. Emerald Amp GT PCR master mix (TaKaRa, Shiga, Japan) were used for PCR reactions.

Sequence analysis

Protein domain and family search were performed using ExPASy Bioinformatics Resource Portal (http://www.expasy.org). PtSAMS and protein sequences of other SAM synthetases of different species including bacterial, fungi, plants, and animals were searched from National Center for Biotechnology Information sequence database (DB) and aligned using Geneious software ver 9.1.6. Phylogenetic tree of PtSAMS protein was constructed by Geneious software ver. 9.1.6. using a neighbor-joining (NJ) method with bootstrap set at 1,000 replicates (Saitou and Nei 1987).

RNA extraction and quantitative real-time PCR

Total RNA was isolated using Hybrid-R RNA purification kit (GeneAll, Seoul, Korea) according to the manufacturer’s instruction. Two micrograms of total RNAs were used for cDNA synthesis using amfiRivert Platinum cDNA synthesis master mix (GenDEPOT, Barker, TX, USA). Quantitative real-time PCR (qRT-PCR) was carried out on Dice Real Time System Single Thermal Cycler (TaKaRa) using SYBR® Premix Ex Taq™ (TaKaRa). PtSAMS gene expression levels in transgenic plants were determined by qRT-PCR using the following primers: PtSAMS-F (5’-GAGGA CCTGATGCCCCCTTGAC-3’) and
PtSAMS-R  
(5’-CTGCGTGCTGATGACAATCG-3’).

AtActin2 gene was used as internal control. AtActin2-F (5’-GATGAGGCGAGTCCAGGAATC-3’) and AtActin2-R (5’-AACCCCAGCTTTTAAGCCTTT-3’) were used for qRT-PCR with the following parameters: 94°C for 30 seconds followed by 40 cycles at 94°C for 5 seconds and 60°C for 30 seconds. Relative expression level was calculated using the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

**Abiotic stress assay**

For Arabidopsis seedling assays, wild type and PtSAMS overexpressing transgenic lines seeds were surface sterilized and germinated on 0.5×MS medium supple-

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Fig. 1. Multiple alignments of S-adenosylmethionine synthetase (SAMS). The deduced amino acid sequence of SAMS of *Pyropia tenera* were aligned with *Populus trichocarpa* (EEE99264.1), *Arabidopsis thaliana* (AAA32868.1), *Gossypium hirsutum* (ADN96174.1), *Arachis hypogaea* (AFN54653.1), *Zea mays* (ACG42196.1), *Oryza sativa* (CAA81481.1), *Nicotiana tabacum* (NP_001312332.1), *Leishmania infantum* (AAB88448.2), *Escherichia coli* str. K-12 subst. MG1655(NP_417417.1), *Saccharomyces cerevisiae* (P10659.2) and Homo sapiens (NP_000420.1). The boxes indicate the binding sites. Blue box: S-adenosylmethionine synthase signature motif (PS00376), [GN]-[AS]-G-D-Q-G-x(3)-G-[FYHG] and red box: S-adenosylmethionine synthase signature 2 (PS00377), G-[GA]-G-[ASC]-[FY]-S-x-K-[DE]. The under lines indicate the conserved domains. Red underline: S-adenosylmethionine synthetase N-terminal domain (PF00438), green underline: S-adenosylmethionine synthetase central domain (PF02772), and blue underline: S-adenosylmethionine synthetase C-terminal domain (PF02773).
mented with different concentrations, 0 mM, 50 mM, 100 mM, or 200 mM of NaCl and KCl, respectively as plant growth condition methods. Seedlings that were completely bleached were considered dead and those with green leaves are considered surviving at 12-day-old plants (Divi et al. 2010). To evaluate the stress tolerance of transgenic lines, plants were vertically grown on the agar plates. Root lengths were compared wild type and PtSAMS-OX. Seedlings were photographed and root lengths were measured (Chen et al. 2014). These experiments were repeated three times.

**RESULTS**

**Amino acid sequence analysis and alignment**

We isolated the SAMS gene from red algae, *P. tenera*. SAMS genes are conserved in various species such as bacteria, fungi, plants, and animals. The function of SAMS has been reported to confer plant organ development. *MAT3* is one of the 4 S-adenosyl methionine synthetase genes in *Arabidopsis* and is required pollen germination and pollen tube growth (Lindroth et al. 2001; Chen et al. 2016). *PcSAMS1* and *PcSAMS2* have been isolated from *Pinus contorta*. *PcSAMS1* is specific to root growth and formation, while *PcSAMS2* functions more generally during plant growth (Lindroth et al. 2001). SAMS from *Panax ginseng* was significantly under salt, salicylic acid, abscisic acid and chilling stresses (Pulla et al. 2009). *PySAMS* gene isolated from *Porphyra yezoensis* was known to be stress related gene. However, its function in plants is unknown.

To better understand the function of PtSAMS, we performed sequence alignment using deduced amino acid sequences of SAMS from *P. tenera, E. coli*, Human, and other species. SAMS are highly conserved among all species tested. Three main domains are highly conserved (Fig. 1). Previous study has reported that two SAMS signature motifs, GAGDQGHMFGY and GGGAFSGKD, are conserved among many species (Hilti et al. 2000; Qi et al. 2009).

![Fig. 2. Amino acid sequence-based phylogenetic analysis of PtSAMS and other species SAMS proteins. Phylogenetic tree was constructed using the Geneious software ver. 9.1.6 with Neighbor-Joining methods. Numbers indicate posterior probabilities values and probability threshold was selected as over 50%. The sequence used for generating phylogenetic tree are *Populus trichocarpa* (EEE99264.1), *Arabidopsis thaliana* (AAAD2868.1), *Gossypium hirsutum* (ADN96174.1), *Arachis hypogaea* (AFN54653.1), *Zea mays* (ACG42196.1), *Oryza sativa* (CAA81481.1), *Nicotiana tabacum* (NP_001312332.1), *Leishmania infantum* (AAB88448.2), *Escherichia coli* str. K-12 substr. MG1655(NP_417417.1), *Saccharomyces cerevisiae* (P10659.2) and *Homo sapiens* (NP_000420.1).]
PtSAMS shares three conserved amino acid sequences with other species. These sequences include the conserved methionine binding motif 17-GHPDK-21, the active signature for ATP binding site 120-GAGDQG-125 which is known as the consensus sequence from ATP binding, and the conserved peptide (Li et al. 2013). SAMS sequences share two SAMS signature motifs: the active signature for ATP binding site 120-GAGDQGIMFGY-130 which is known as the consensus sequence for ATP binding and the conserved peptide 267-GGGAFSGKD-275 which forms a P-loop for the phosphate-binding region.

Phylogenetic analysis of SAMS was performed with Geneious software ver 9.1.6 using NJ method. Results are shown in Fig. 2. Based on SWISS-MODEL spatial structure analysis, the enzyme monomer had three domains: the N-terminal domain, the central domain, and the C-terminal domain (Fig. 3). In addition, PtSAMS was predicted to have two substrate binding sites: a site for ATP binding between the central and C-terminal domains and a Met binding site between the central and N-terminal domain (Fig. 3) (González et al. 2000).

**Production and analysis of 35S::PtSAMS transgenic plants**

To elucidate the function of PtSAMS in plants, PtSAMS gene was isolated from cDNA library of *P. tenera* using RT-PCR and introduced into a pPZP vector consisting of 35S CaMV promoter, PinII terminator, and *bar* genes (Fig. 4A). We generated T0 pool by floral dipping method in *Arabidopsis*. To select transgenic plant, T0 seeds were grown on PPT containing 0.5×MS media. After extraction of genomic DNAs from selected T1 transgenic plants, PCR

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**Fig. 3.** Analysis of three-dimensional structure of the PtSAMS protein by Phyre programs. The 3-dimensional model of PtSAMS protein was generated on the basis of the protein data bank (PDB) template of Homo sapiens S-adenosylmethionine synthetase protein (PDB ID: c2obvA). Blue arrow (S-adenosylmethionine synthase signature modif; PS00376), 120-GAGDQGIMFGY-130 and red arrow (S-adenosylmethionine synthase signature modif 2; PS00377), 267- GGGAFSGKD-275. Yellow arrow (methionine binding motif): 17- GHPDK-21 and green arrow (ATP biding site): 120-GAGDQG-125. The amino and carboxylic terminal are labeled N and C, respectively. Each SAM synthetase domains are marked with white line.

ATP: adenosine triphosphate.

**Fig. 4.** Schematic representation of the expression vector constructs for PtSAMS overexpression lines. (A) Constructs used for the overexpression of PtSAMS, for which each full length cDNA was cloned into the plant expression vector that harbored a phosphinothricin (PPT) resistance gene. (B) Polymerase chain reaction analysis of 35S::PtSAMS transgenic T1 plants by the bar gene specific primer. M: marker, WT: wild type (Ler), lane 3-11: transgenic plants (T1). (C) Detection of *bar* gene expression of 35S::PtSAMS transgenic plants using anti-Bar immune strip.

RB: right border, LB: left border, OX: overexpression, M: marker, WT: wild type (Ler).
analysis and Bar immune strip test were performed to select transgenic plants that possessed the bar gene (Fig. 4B, C). We have obtained 9 transgenic lines. Afterward, the selected two T1 plants were used to produce the T2 generation to separate homozygous lines for further analysis.

**Expression of PtSAMS gene in transgenic plants**

To verify the transcriptional levels of PtSAMS gene, transgenic lines were subjected to qRT-PCR analysis. 35S::PtSAMS #4 and #16 showed higher levels of expression compared to wild type (Fig. 5). 35S::PtSAMS #3, #5, #6, and #15 showed relative lower levels of expression compared to the two transgenic lines #4 and #16. Therefore, these two lines were used in abiotic stress assays.

**Analysis of abiotic stress tolerance**

To investigate the effect of PtSAMS on Arabidopsis under abiotic stress, seeds of wild type and transgenic lines were germinated on 0.5×MS medium supplemented with 0 mM (normal condition), 50 mM, 100 mM, or 200 mM of NaCl and KCl. Growth patterns of both wild type and transgenic lines were observed at 12-day-old plants and root length were measured (Fig. 6). The experimental

![Fig. 5. Analysis of the transgene expression in the transgenic plants. Expression levels of the PtSAMS gene in transformants. AtActin2 gene was used as internal control. Quantitative real-time polymerase chain reaction was analyzed via the 2-ΔΔCT method. The results are presented as average values with standard deviation using each three times. WT: wild type (Ler).](image)

![Fig. 6. Comparison of the growth of wild type and transgenic lines overexpressing the PtSAMS gene under salinity stress. (A) The growth was assayed on medium containing 0 mM, 50 mM, 100 mM, or 200 mM NaCl and KCl from the top. The growth pattern was analyzed within 12 days after sowing in medium containing each concentration of NaCl and KCl. (B) Root length was measured 12 days after seeding. This experiment was repeated three times. Bar: 2 cm. WT: wild type (Ler).](image)
results revealed that there was no significant difference in growth patterns and root length from 12-day-old plants between transgenic lines and wild type under abiotic stress conditions (Fig. 6).

**DISCUSSION**

SAM is an important molecule that plays a central role in many cellular biological reactions and methionine metabolism (Martínez-López *et al.* 2008). Biosynthesis of SAM is catalyzed by SAMS using methionine and ATP (Shen *et al.* 2002). SAMS is also highly conserved throughout the eukaryotic and prokaryotic kingdoms. PtSAMS consists of three domains for S-adenosyl-lmethionine synthesis. It has two substrate binding sites with representative features (Fig. 1, 2).

Previous studies have shown that SAMS genes have differential expression patterns in *Arabidopsis* such as roots, stems, pea ovaries, and rice leaves. In addition, SAMS gene can influence adventitious root development in black locust. MAT3, an S-adenosylmethionone synthetase expressed in pollen, plays an important role during pollen germination and pollen tube growth (Chen *et al.* 2016).

SAMS catalyzes the synthesis of SAM, a precursor of ethylene, and polyamine (PA) biosynthesis (Wawrzynska *et al.* 2015). Ethylene mainly regulates germination, adventitious root formation, growth, development, and senescence in plants (Roy *et al.* 1972; Ge *et al.* 2006). PAs are ubiquitously present in nature. They are required for cell growth (Fujita *et al.* 2012). Increase in endogenous amounts of PAs can be induced by environmental stresses including osmotic pressure, temperature, and pH (Pang *et al.* 2007). Furthermore, oxidative stress can induce PA biosynthesis and PA content including spermidin and spermine (Smirnova *et al.* 2012). Some reports have revealed that overexpression of SAMS gene can enhance the tolerance of tobacco against abiotic stress and increase the PA content in tobacco (Luo *et al.* 2016).

Overexpression of *Medicago SAMS* gene can also enhance salt tolerance and strength roots (Hua *et al.* 2012). Besides, upregulation of *Chorispora bungeana SAMS* involves the content of PAs to deal with cold and high salinity stress (Ding *et al.* 2015).

To examine the function of PtSAMS in plants, we generated transgenic plants of *Arabidopsis* (Fig. 4). Salinity stress assay was carried out in wild type and selected transgenic lines plants after treatment with different concentrations of NaCl and KCl. However, there was no significant difference in germination rate or growth pattern between wild type and PtSAMS-OX transgenic line (Fig. 6). To investigate the role of PtSAMS under salinity stress tolerance, germination rate was observed at 12-day-old plants after 0 mM, 50 mM, 100 mM, or 200 mM NaCl and KCl treatment, respectively. No difference was observed between wild type and transgenic plant lines (#4 and #16) in the growth patterns and root length under salinity stress treatment. One of the reasons suggests that PtSAMS gene does not function in plants because of difference of sequence similarity between *Arabidopsis* and red algae. Another possibility is that SAMS gene may play a role the organ development like OsSAMSs and AtSAMSs in plants (Lucena *et al.* 2015). Therefore, to better understand the function of PtSAMS gene, further studies are needed to analyze plant development and growth.

**ACKNOWLEDGEMENTS**

This study was supported by a grant (2016M00500) funded by National Marine Biodiversity Institute of Korea Research Program. It was also supported by a grant (Project No. PJ0118292016) from the Next-Generation Biogreen 21 Program funded by Rural Development Administration, Jeonju, Republic of Korea.

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