



Cloning, expression, and enzymatic activity analysis of strawberry

S-adenosyl-L-methionine synthetase gene *FaSAMS1*

Shu-Fang Wang¹, Cheng Xu¹, Xiaofeng Liu¹, Yuanyue Shen*

Beijing Key Laboratory of New Technology in Agricultural Application, College of Plant Science and Technology, Beijing University of Agriculture, No.7 Beining Road, Changping District, Beijing 102206, People's Republic of China.

This work was supported the China National Science Foundation (Project 31272144, 31040006, 41473004) and the National Key Technology Supported Program of China (Project 2011BAD32B03), and the Project of Construction of Innovative Teams and Teacher Career Development for Universities and Colleges under Beijing Municipality (grant no. IDHT20140509).

Abstract

The S-adenosyl methionine synthetase gene *FaSAMS1* is involved in the regulation of strawberry fruit ripening, however, the biochemical properties of *FaSAMS1* protein remain unclear. Here, a coding cDNA sequence of *FaSAMS1* was cloned by RT-PCR and inserted into a recombinant yeast expression vector pPICZA, then transformed into the yeast expression strain X-33. The fusion protein *FaSAMS1* was induced, expressed, and purified. The enzymatic activity analysis of *FaSAMS1* showed that the reaction system contains 0.09 mg of *FaSAMS1*, protein concentration reached at 0.454 mg·mL⁻¹, the activity of the enzyme was 0.32 U, specific activity was 3.56 U·mg⁻¹. This study has provided biochemical evidence for the involvement of ethylene in the regulation of strawberry fruit ripening.

Keywords: Strawberry; fruit ripening; S-adenosyl-L-methionine synthetase (SAMS); yeast expression

Introduction

The gaseous plant hormone ethylene plays an important role in various aspects of plant growth and development, as well as environmental stress (Fluhr and Mattoo, 1996; Wang et al., 2002). Especially, much progress has been made in understanding the molecular mechanisms of ethylene in the regulation of climacteric fruit ripening using the model plant tomato (Klee and Giovannoni, 2011). Notably, in recent years, ethylene is also involved in the regulation of non-climacteric fruit ripening using the model plant strawberry (Sun et al., 2013), demonstrating that downregulation of the ethylene biosynthesis-related gene *FaSAMS1* inhibits ripening. However, the biochemical nature of the *FaSAMS1* protein remains yet unknown.

The ethylene biosynthesis in model plant *Arabidopsis* are well defined (Thomas and Surdin-Kerjan, 1991; Kende, 1993; Bleecker and Kende, 2000): (1) conversion of methionine to S-adenosyl-L-methionine (SAM) catalyzed by SAM synthetase (SAMS), (2) formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM via ACC synthase (ACS) activity, and (3) conversion of ACC to ethylene through ACC oxidase (ACO). Owing to a critical role of ethylene in triggering tomato fruit ripening, to a large extent, this model has also been further developed in the fruit (Barry and Giovannoni, 2007), finding that ACC synthase *LeACS2* and ACC oxidase *LeACO1* control tomato fruit ethylene production and tomato ethylene receptor *tCTR1* is induced by both



ripening and exogenous ethylene (Klee, 2002). Downregulation of *FaSAMS1* or *FaCTR1* transcripts not only inhibits strawberry fruit red-coloring, but also applied ethephon could promote natural strawberry fruit red-coloring and softening, as well as rescues partially anthocyanin biosynthesis in the RNAi fruit, demonstrating that ethylene is required for strawberry fruit ripening (Sun et al., 2013). Although SAM synthetase committed the first step in ethylene biosynthesis is proved to play a role in strawberry fruit ripening (Sun et al., 2013), its biochemical properties of FaSAMS1 protein remain unclear.

In the present study, fusion protein FaSAMS1 was induced, expressed, and purified using prokaryotic expression system. Enzymatic activity analysis of FaSAMS1 showed that the specific activity of FaSAMS1 reached to 3.56 U·mg⁻¹, demonstrating that strawberry SAM synthetase FaSAMS1 has high enzymatic activity. This study has provided biochemical evidence for the involvement of ethylene in the regulation of strawberry fruit ripening.

Materials and methods

Plant Material

Strawberry (*Fragaria × ananassa* 'Fugilia') fruit was used. Strawberry plants were grown in a greenhouse (20°C–27°C, relative humidity of 70% - 90%, 15 hr/ 9 hr light/dark cycles) during spring seasons from 2014 to 2015. Six uniformly-sized red fruit were sampled, and quickly stored by liquid nitrogen for RNA isolation.

Cloning of the *FaSAMS1* gene

The cDNA obtained above was used as a template for amplifying the *FaSAMS1* gene with primers (forward, 5'-ATGGAGACTTTCCTATTCACATCTG-3'; reverse, 5'-TTAAGACTGAGGCTTCTCCCAC-3'). PCR was performed under the following conditions: 94°C for 5 min, followed by 32 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for an additional 5 min. The PCR products were ligated into a T1 simple vector and subsequently transformed into *TransI-T1*. Positive colonies were selected, amplified, and sequenced by Invitrogen China (Shanghai, China).

Expression and purification of *FaSAMS1* recombinant protein

The expression and purification of the recombinant protein FaSAMS1 was used EasySelect™ Pichia Expression Kit and ProBond™ Purification System (Invitrogen, USA), respectively. The coding sequence of *FaSAMS1* was amplified by PCR from a cDNA synthesized above using primers [forward, GGAATTCAAAAATGGAGACT

TTCCTATTCACATCTG (*EcoRI* site underlined); and reverse, GGGGTACCAGACTGAG GC TTCTCCCAC (*KpnI* site underlined)] and cloned into the expression vector pPICZ A in frame with the C-terminal tag fusion, which was transformed into *E. coli* to select transformants on Low Salt LB plates containing 25 µg/mL Zeocin™. Twenty transformants were selected to confirm the frame fusion with the C-terminal tag by sequencing. The purified and linearized recombinant plasmids were transformed into *Pichia pastoris* X-33 to select the Zeocin™-resistant yeast transformants by YPDS plates containing the appropriate concentration of Zeocin™. The FaSAMS1 fusion protein was expressed and purified by Ni-NTA His Bind Resins according to manufacturer's protocols. The eluted fusion protein was stored at -80°C until use.

The analysis of enzyme activity

The SAM enzyme activity assay uses enzyme catalysis. Enzyme activity standard curve was made. S-adenosyl methionine (5.5 mg) was formulated as the concentration of mother liquid 1.1 mg / ml. Taking 800 µl of mother liquor diluted to 5 ml as standard 1, then we diluted 2 times of standard 1 as standard 2, standard 3, 4, and 5 were made by using twice gradient dilution method above. Take these five samples draw the S-adenosyl methionine standard curve. To determine the enzyme activity, enzyme catalyzed reaction of substrate ATP and L-methionine carried out in the buffer, the amount of enzyme reaction product of SAM was detected. Enzyme reaction system included ATP (final concentration of 1.5 mM) 2 µl, Met (final concentration of 150 µM) 5 µl, buffer solution 93 µl, 100 µl recombinant strain. To each tube, we added 500 ml of the substrate mixture, which contained 100 mM Tris (pH 9.0), 20 mM MgCl₂, 150 mM KCl, and 5 mM of DTT. The tubes were then incubated at 37 °C for 10 min. After the end of enzyme reaction, reaction liquid was centrifuged in 10,000 rpm for 20 min. The supernatant (1 ml) was used for measuring the amount of product S-adenosylmethionine (S-adenosylmethionine in 254 nm has the maximal light absorption).

Results

Cloning of *FaSAMS1* gene from strawberry fruit

To clone the *FaSAMS1* gene, an *Arabidopsis* SAMS1 protein was used to BLAST in a strawberry gene library (<https://strawberry.plantandfood.co.nz/index.html>), and a high homology protein with the gene was found in the 24,556 position of the gene locus. Based on the nucleotide sequence, specific primers (forward, 5'-ATGGAGACT TTCCTATTACATCTG-3'; reverse, 5'-TTAAGACTGAGGCTTCTCCCA

C-3') were designed to amplify a coding sequence of the *FaSAMS1* gene from strawberry fruit. The results showed that an 1,182 bp cDNA homologous to the *Arabidopsis* gene SAMS1 (named *FaSAMS1*) was isolated from strawberry fruit through RT-PCR. The cDNA included an open reading frame that encodes a deduced protein of 393 amino acids, in which the putative conserved domains were detected by homology analysis using BLAST for the *FaSAMS1* protein on the NCBI website, <http://blast.ncbi.nlm.nih.gov/Blast.cgi> (Fig. 1), suggesting that the putative strawberry potassium channel gene *FaSAMS1*, was cloned successfully.

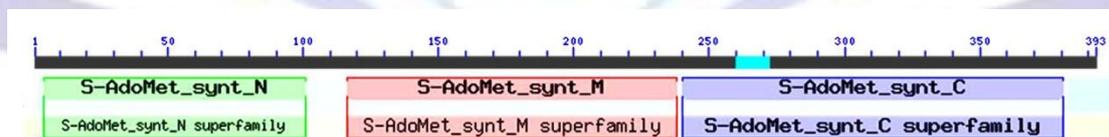


Fig.1 Conserve domains of S-adenosyl-L-metnionine synthetase in *FaSAMS1*

Construction and expression of *FaSAMS1* fusion protein

The *FaSAMS1* cDNA sequence (1,182 bp) was inserted into the yeast expression vector PpicZA by *EcoR1* and *Kpn1*. The recombinant plasmid was extracted and identified by double enzyme digestion with *EcoR1* and *Kpn1*. The *FaSAMS1* gene was successfully constructed and expressed in a yeast expression vector (Fig. 2A, arrow). Expression vector containing *FaSAMS1* gene was electroporated into yeast strain to express *FaSAMS1* fusion protein. The results of electrophoresis for purified protein demonstrated that 55 kD fusion protein was collected by affinity chromatography with His tag (Fig 2B).

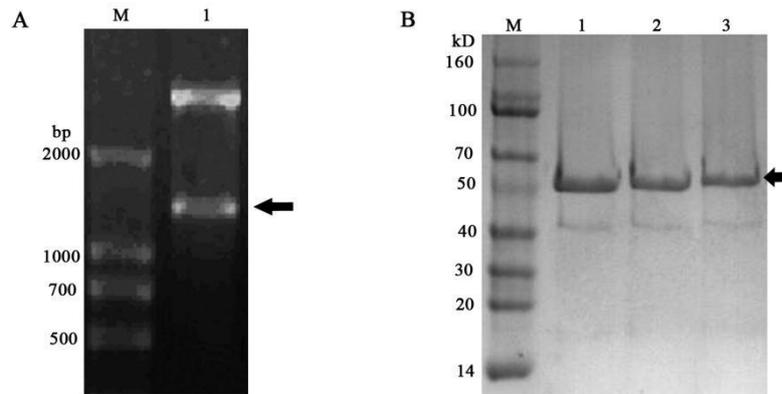


Fig.2 Identification of FaSAMS1 recombinant plasmid and purification of the fusion protein.

A: Endonuclease digestion. M: Marker, 500-2000 bp; 1: Digestion of recombinant plasmid; B: SDS-PAGE identification of the purified protein; M: Marker, 14-160 kD; 1, 2 and 3: 1, 2 and 3 elution tubes.

Enzymic activity analysis of FaSAMS1

In order to determine the activity of FaSAMS1, the chemical reaction and spectrophotometry were used in this study. L-methionine generated S-adenosylmethionone under the reaction of S-adenosylmethionine synthetase. S-adenosylmethionine in 254 nm has the maximal light absorption, depending on the amount of S-adenosylmethionine after enzymatic reactions to determine of SAMS activity by standard curve (Fig. 3). The results demonstrated that the reaction in 37°C for 10 min generated S-adenosylmethionine with the concentration of $0.316 \text{ nmol}\cdot\text{ml}^{-1}$, FaSAMS1 protein concentration was $0.454 \text{ mg}\cdot\text{ml}^{-1}$, the measured enzyme activity was 0.32 U and the specific activity was $3.56 \text{ U}\cdot\text{mg}^{-1}$.

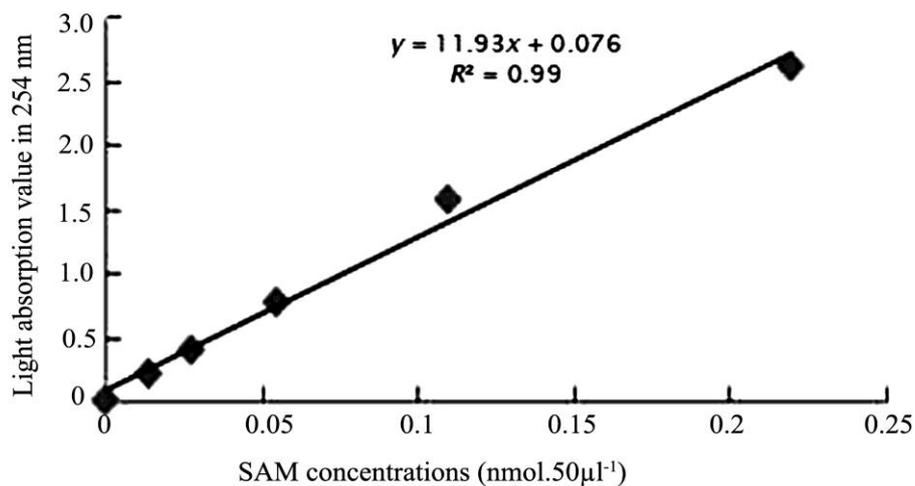


Fig. 3 The criterion curve of standard SAM.

Discussion

The expression of foreign proteins in yeast expression system has post translational modification, which makes the expressed protein with native activity. Thus, yeast expression provides a powerful



tool for the identification of protein biochemical functions. In the present study, *FaSAMS1* gene was integrated into yeast expression vector pPICZA and then the recombinant plasmid was transformed into X-33 yeast strain. The fusion protein was induced by methanol and the activity of this fusion protein was analyzed by enzymatic reaction and spectrophotometry. Our results demonstrated that the expressed protein had high enzymatic activity, reaching to specific activity at $3.56 \text{ U} \cdot \text{mg}^{-1}$.

It is previously reported that downregulation of *FaSAMS1* transcripts inhibits fruit red-coloring, and furthermore, exogenous ethephon could rescue anthocyanin biosynthesis in the RNAi fruit, demonstrating that *FaSAMS1* plays a role in the regulation of strawberry fruit ripening (Sun et al., 2013). Although SAM synthetase committed the first step in ethylene biosynthesis is proved to play a role in strawberry fruit ripening (Sun et al., 2013), the biochemical properties of *FaSAMS1* protein remain unclear. In the present study, it is confirmed that *FaSAMS1* had high enzymatic activity. This study has provided biochemical evidence for an important role of ethylene in the regulation of strawberry fruit ripening.

References

1. Barry CS, Giovannoni JJ. 2007. Ethylene and Fruit Ripening. *J Plant Growth Regul* 26: 143-159.
2. Bleecker AB, Kende H. 2000. Ethylene: a gaseous signal molecule in plants. *Ann Rev Cell and Dev Bio* 16: 1-40.
3. Fluhr R, Mattoo AK. 1996. Ethylene biosynthesis and perception. *Crit Rev in Plant Sci* 15: 5-6.
4. Kende H. 1993. Ethylene biosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* 44: 283-307.
5. Klee HJ. 2002. Control of ethylene-mediated processes in tomato at the level of receptors. *J Exp Bot* 53: 2057-2063.
6. Klee HJ, Giovannoni JJ. 2011. Genetics and control of tomato fruit ripening and quality attributes. *Annu Rev Genet* 45: 41-59.
7. Sun JH, Luo JJ, Tian L, Li CL, Xing Y, Shen YY. 2013. New evidence for the role of ethylene in strawberry fruit ripening. *Journal of Plant Growth Regulation* 32: 461-470
8. Thomas D, Surdin-Kerjan Y. 1991. The synthesis of the two S-adenosyl-methionine synthetases is differently regulated in *Saccharomyces cerevisiae*. *Mol Gen Genet* 226: 224-232.
9. Wang K, Li H, Ecker J. 2002. Ethylene biosynthesis and signaling networks. *Plant Cell* 14: S131-151