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Characterization of Plant S-Adenosyl-L-Methionine Decarboxylase and Spermidine Synthase in Polyamine Deficient Mutant Strain of *E. Coli*

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Abstract

S-adenosylmethionine decarboxylase (SAMdC, EC 4.1.1.50) and Spermidine synthase (SPDS, EC 2.5.1.1), two enzymes of plant polyamine biosynthetic pathway were cloned to gain further insight on the polyamine metabolism through a molecular approach. cDNAs for SAMdC and SPDS were isolated from Oryza sativa cv. Pokkali and Nicotiana tabacum cv. Jayasri respectively. Rice SAMdC (rSAMdC) and Tobacco SPDS (tSPDS) proteins were overexpressed in E. coli using expression vectors pEZZ18 and pGEX-3X respectively. N-terminally protein-A-tagged 43.8 kDa pre-protein of rSAMdC and N-terminally GST-tagged 34.7 kDa of tSPDS were purified by affinity chromatography. The activities of the recombinant proteins were confirmed by the appearance of spermidine, product of the coupled reaction involving rSAMdC and tSPDS. As a direct evidence of the function of these plant enzymes, the complementation assay using extract of E. coli mutant strain, HT252 (F-Δ(speAspeB) Δ(speCglc) Δ(speED) thr-1 proA2 thi-1 lacY1 galK2 m-), deficient in polyamine biosynthetic enzymes was performed. Reconstitution of the E. coli polyamine biosynthetic pathway by two different plant enzymes rSAMdC and tSPDS, simultaneously supplemented with exogenous S-adenosylmethionine and putrescine, was the novel observation of the present work.

Keyword: *E.coli* Polyamine Biosynthetic Mutant HT252, S-Adenosyl-L-methionine decarboxylase, Spermidine Synthase.

Introduction

Polyamines like putrescine, spermidine and spermine are omnipresent in all living organisms. Polyamines take part in a wide range of core processes at the cellular and molecular levels of biological system[1,2]. The pathway of polyamine biosynthesis has been established in many organisms. In fungi and mammals, putrescine is produced from ornithine through a single catabolic pathway by ornithine decarboxylase (ODC, EC 4.1.1.17). An additional route for putrescine production is also established in plants and in some bacteria, where putrescine is produced from arginine by the activity of arginine decarboxylase (ADC; EC 4.1.1.19). Consequently, spermidine is synthesized from putrescine by addition of an aminopropyl group, which is donated by decarboxylated S-adenosylmethionine (dcSAM). The conversion of S-adenosylmethionine (SAM) to decarboxylated S-adenosylmethionine (dCSAM) is mediated by S-adenosylmethionine decarboxylase (SAMdC) (EC 4.1.1.50). Decarboxylated S-adenosylmethionine (dCSAM) combines with putrescine with the help of spermidine synthase (SPDS, EC 2.5.1.16) to produce spermidine. Finally, spermine is produced when dCSAM combines with spermidine by spermine synthase (SPMS, EC 2.5.1.22).

It has been widely reported that in a variety of plants the level of polyamines increase due to adaptation to stresses. The indispensable role of polyamine in stress tolerance is well documented[1]. In cereals and pulses polyamines act as one of the major abiotic stress hindering molecules[3,4]. Furthermore, exogenous polyamines particularly spermidine / spermine has been shown to alleviate salinity stress induced damage by accumulation of Na^+, loss of K^+ and loss of chlorophyll in rice plants[5].

SAMdC is a key factor involved in biosynthesis of higher polyamines viz. spermidine and spermine by the synthesis of dcSAM. Under physiological condition the concentration of dcSAM is very low and its availability may actually control the rate of higher polyamine production.

Manipulating the endogenous polyamines pools applying transgenic technology is an important tool for studying their physiological roles in plants[6,7]. Over expression or down regulation of ADC, ODC and SAMdC have shown modulation of the cellular polyamine pool[7]. Putrescine accumulation was reported from the transgenic plants over expressing heterologous ADC or ODC cDNA[8, 9]. The negative feed back mechanism to control ADC, ODC and SAMdC enzyme activity by polyamine has been well established. Over expression of oat ADC cDNA in tobacco plants showed distinct rise of transgenic transcript without change in endogenous polyamine pool indicating a strong regulatory mechanism of polyamine biosynthetic pathway[10]. On the other hand transgenic tobacco over expressing mouse ODC cDNA, showed increased

putrescine accumulation[10]. In a similar experiment with rice, overexpression of oat ADC cDNA caused no significant increase of ADC enzyme activity and polyamine accumulation in vegetative tissue. This provides a strong evidence for the presence of a tight regulation at the level of ODC / ADC in tobacco and rice plants[10,11].

Heterologous expression of SAMdC and SPDS genes were reported in several systems, such as, over expression of *Tritordium* SAMdC in rice[12], over expression of human SAMdC in tobacco[12], over expression of carnation SAMdC in tobacco and enhanced stress tolerance[13]. Transgenic *Arabidopsis thaliana*, *Ipomea batatas* and pear over expressing SPDS gene showed multiple environmental stress tolerance[12]. Moreover, a positive correlation between the SAMdC transcript level and spermidine production during development was reported in *Arabidopsis*. This indicates that a combined activity of SAMdC and SPDS is responsible for spermidine production[14]. These observations suggest that the gene manipulation strategy involving SAMdC and SPDS could be effective to impart environmental stress tolerance *in planta*.

Despite numerous studies on the heterologous expression of these enzymes, clear-cut data for the polyamine's mechanism of action is yet to reveal. Manipulation of the endogenous levels of polyamines may provide definite clue[10]. Moreover, manipulation of one enzyme of a biosynthetic pathway is not sufficient to develop a clear insight of it. Franceschetti et al[10] have also shown that the overproduction of SPDS enzyme alone failed to produce high levels of higher polyamine in total. The production of spermidine has been shown to be synchronized by the accessibility of the substrates i.e. putrescine (Put) and dcSAM and the level of SPDS activity.

The present study, hence, was envisaged for heterologous expression of both SAMdC and SPDS simultaneously in *E. coli* mutant HT252 (*F- Δ(speAspeB) Δ(speCglc) Δ(speED) thr-1 proA2 thi-1 lacY1 galK2 m-*), deficient in polyamine biosynthetic pathway. To determine the functional activity of the enzymes by the reconstitution of the polyamine biosynthetic pathway, the *E. coli* mutant strain was used as a tool.

Material and Methods

Cloning of cDNAs: Roots of salt treated (150 mM NaCl, 16 h) 10 days old rice seedlings was used for the isolation of total RNA from rice seedlings (*Oryza sativa* cv. Pokkali) by GITC method[15]. PolyA$^+$ RNA was prepared using Oligo dT-cellulose column (Pharmacia) following the protocol recommended by the manufacturer. First strand cDNA was synthesized from 1 µg of polyA$^+$ RNA, using 'Superscript RT' reaction kit from GIBCO BRL, according to the company's protocol. A pair of PCR primers (table 1, figure 1A) were designed from the extreme 5' and 3' ends of *Oryza sativa* SAMdC cDNA sequence (Gen Bank accession. no. YO7766)[16].First strand cDNA was amplified by PCR using 100 ng each of gene specific primers, rSAM5 and rSAM3, 10 ng of first strand cDNA, 0.2 mM dNTP, 2.5 U of Platinum Taq High Fidelity (Life Technologies) and 1 mM $MgCl_2$. PCR reaction was done in a thermocycler (Perkin Elmer, 2400) by running 10 cycles of 1 min at 94°C, 1.5 min at 58°C and 1 min at 72°C, followed by 20 cycles of 1 min at 94°C, 1.5 min at 52°C and 2 min at 72°C with a final extension of 15 min at 72°C. A PCR product (1,197 bp) containing the full-length rSAMdC cDNA was obtained. For easy subcloning into plasmid vector, BamH1 restriction site was introduced in both 5' and 3' primers. The PCR product was subcloned into the pBluescript vector at BamHI site and transformed into DH5α competent cells. Recombinant plasmid DNA was isolated and digested with the restriction enzyme BamHI to release the rSAMdC insert DNA, for cloning into pEZZ18 (Amersham Pharmacia) *E. coli* expression vector. The expression of the pEZZ18 is controlled by the lacUV5 and Protein-A promoter and it is inducible only by high temperature exposure.

SPDS cDNA was amplified by RT-PCR from total RNA isolated from young leaves of three months old tobacco plants (*Nicotiana tabacum* cv. Jayasri)[15]. A pair of gene specific PCR primers (table 1, figure 1B) were designed from the extreme 5' and 3' ends of tobacco SPDS cDNA sequence (GenBank accession no. AB006692)[17]. The first strand cDNA was synthesized from 1 µg of total RNA with 'Thermoscript Reverse Transcriptase' reaction kit from Life Technologies, under the conditions recommended by the manufacturer. PCR reaction mixtures containing 100 ng each of two gene-specific primers (tSPDS5 and tSPDS3), 10 ng of first strand cDNA, 0.2 mM dNTP and 2.5 U of Platinum Taq High Fidelity (Life Technologies) and 1 mM $MgCl_2$ were incubated in a thermocycler (Perkin-Elmer, 2400) for 5 min at 94ºC followed by 30 cycles of 1 min at 94ºC, 3 min at 72ºC and finally a 15 min extension at 72ºC. Gene specific primers tSPDS3 and tSPDS5 were designed according to *Nicotiana sylvestris* spermidine synthase cDNA sequence by Hashimoto et al[17] (Genbank accession no. AB006692). The amplified PCR product of 950 bp was purified by QIAquick PCR purification Kit (Qiagen), subcloned in pBluescript at the EcoR1 site and transformed into DH5α competent cells. An insert of 950 bp was obtained by digestion with *EcoR1*enzyme from recombinant plasmid. Both the cDNAs were sequenced by the dideoxy method using T7 and T3 primers and also by gene specific primers from two ends[18].

Sub cloning in the *E.coli* expression vector and purification of the expressed protein: Complete cDNA sequence of rSAMdC (1,197 bp) was excised as a BamH1 fragment from pBluescript. It was subsequently sub-cloned into the BamH1 site of pEZZ18 expression vector to produce Protein-A tagged fusion protein[19]. *E. coli* strain HB101 carrying the pEZZ:rSAMdC was grown overnight at 37°C in LB liquid medium. The fresh subcultured cells (1:100 dilutions) were grown at 37ºC for 2 h, until the OD_{600} of the culture reached 0.6 and then for 4 h at 42ºC to induce Protein-A expression. The

cells were sonicated (Cole-palmer) in 10 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.1% Bovine Serum Albumin, 0.1% Triton X-100, 0.1% SDS, and 0.1% Tween-80 and centrifuged for 30 min at 10,000 rpm at 4ºC. IgG sepharose affinity resin was used for the purification of fusion protein following the protocol of manufacturer (Amersham Pharmacia). Fusion proteins, eluted with 0.1 M Citrate buffer (pH 3.0) were subjected to SDS-PAGE (12%) analysis.

Complete cDNA sequence of tSPDS (950 bp) was excised as EcoR1 fragment from pBluescript and sub-cloned into the EcoR1 site of pGEX-3X expression vector (Amersham Biosciences) to produce a GST fusion protein. The recombinant plasmid (pGEX-3X: tSPDS) was transformed into *E.coli* strain BL21 and grown overnight at 37ºC. The freshly sub-cultured cells (1:100 dilutions) were grown until the OD_{600} of the culture reached 0.6 and then grown with 1 mM IPTG for 4 h at 37ºC to get maximum induction. The cells were harvested. The pellet was resuspended in lysis buffer containing 50 mM Tris.Cl (pH 8.0), 1 mM EDTA, 100 mM NaCl (3 ml / gm cell pellet), 8 µl of 50 mM Phenyl Methane Sulphonyl Fluoride (PMSF) / gm cell pellet, and sonicated. The fusion protein was purified by glutathione sepharose 4B (Pharmacia) affinity column. The elution buffer contained 10 mM glutathione in 50 mM Tris HCl pH 8.0. From the fusion protein, factor Xa was removed by restriction protease factor Xa cleavage and removal kit (Roche). The fusion protein was dissolved in 50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM $CaCl_2$. Lyophilised factor Xa dissolved in HPLC grade water was incubated to the fusion protein at a ratio of 1/100 at 4°C for 16 h. For removal of the factor Xa, streptavidin gel was incubated with cleavage sample at 4°C for 60 min with shaking. Streptavidin bound factor Xa was removed by centrifugation. The digested products were analyzed by 12% SDS-PAGE.

Table-1
Oligonucleotides used to amplify the cDNAs for both the enzymes from rice and tobacco leaves

No	Name of cDNA	Oligonucleotide primers	Name of the primers
1.	S-adenosy-L-methionine Decarboxylase	5'-CATGGATCCAACAATGGGAG ACTTGTGTGCTGCTGACCC-3'	rSAM5
2.	S-adenosy-L-methionine decarboxylase	5'-CTCGGATCCTATTACTCCTA ATCAAACACCCTATCATTC-3'	rSAM3
3.	S-adenosy-L-methionine Decarboxylase	5'CTTCCTGAGGAAGTTGCT3'	rSAM5A (IP)
4.	Spermidine synthase	5'- CTGAATTCATCTAGAACAATGGAA GCAGCAAACCACAACAACAACGG3'	tSPDS5
5.	Spermidine synthase	5'-GATGAATTCTCTAGATCATTTTCC TTTGGATTCAATCACCCTCTTGGC3'	tSPDS3

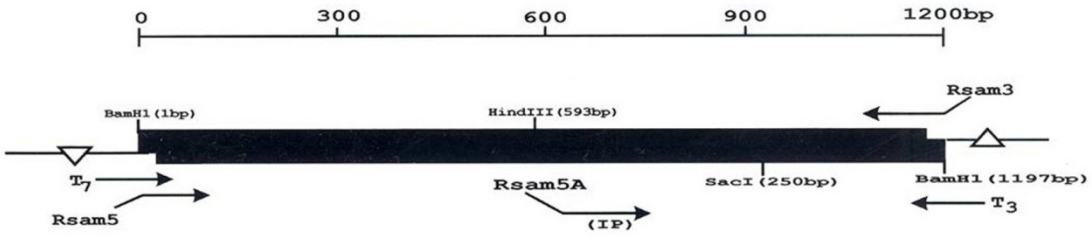

Figure 1A

Figure 1B

Figure-1
Molecular cloning of rice SAMdC and tobacco SPDS cDNA : Cloning of cDNAs for S-adenosyl-l-methionine decarboxylase from salt treated rice seedlings (figure 1A) and for Spermidine synthase from tobacco leaves (figure 1B) by gene specific primers (table 1); both the cDNAs were cloned in pBluescript vector and sequenced by first T7 and T3 primers and then by gene specific primers

Characterization of the expressed protein: To test the functional activity of the enzymes rSAMdC and tSPDS, pEZZ:rSAMdC or pGEX-3X:tSPDS were transformed to complement the mutant *E. coli* strain HT252 (*F- Δ(speAspeB) Δ(speCglc) Δ(speED) thr-1 proA2 thi-1 lacY1 galK2 m-*), in which arginine decarboxylase, ornithine decarboxylase, agmatinureo hydrolase, S-adenosylmethionine decarboxylase and spermidine synthase genes were mutated. To study the activity of the expressed protein rSAMdC, recombinant HT252 *E. coli* cells containing pEZZ:rSAMdC were inoculated (from overnight culture) in fresh LB liquid medium[17] and grown upto OD_{600} of 0.6 and further grown for 4 h at 42ºC. The cells were harvested by centrifugation and washed twice with Phosphate-buffered saline (pH 7.4) to remove extra cellular medium. The harvested cells were sonicated and centrifuged at 10,000 rpm for 10 min at 4ºC. Recombinant HT252 *E. coli* cells containing pGEX-3X:tSPDS were inoculated (from overnight culture) in fresh LB liquid medium[18] and grown upto OD_{600} of 0.6 and further grown for 4h at 37ºC with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). Cells were harvested by centrifugation, washed with Phosphate-buffered saline (pH 7.4) and the sonicated. The cell lysate containing 100 µg of pEZZ:rSAMdC protein supplemented with 5 mM SAM and 5 mM Putrescine separately or together were incubated at 37°C for 30 min. The cell lysate of pGEX-3X: tSPDS containing 100 µg of protein or 5 µg of the purified enzyme was added to the reaction mixture separately.

To study the activity of these two enzymes, synthesis of spermidine was measured by dansylation and Thin Layer Chromatography (TLC) on high-resolution silica gel HPTLC plate (Silica gel 60F254 MERCK). Spermidine was quantified by UV spectrofluorometer (Perkin-Elmer, MmPF 44B) at an excitation wavelength of 360 nm and an emission wavelength of 506 nm[20].

Results and Discussion

Molecular cloning of Rice SAMdC and Tobacco SPDS cDNA: A PCR product (1,197 bp) containing the full-length rSAMdC cDNA was obtained. In rice genome SAMdC 1 gene is present as a single copy sequence. When subjected to salinity stress a steady accumulation of the SAMdC1 transcript was reported in rice plants[21]. The sequence obtained was compared with the sequence of Rice SAMdC1 (accession. no. YO7766) and Rice SAMdC2 cDNA sequences (accession. no.AJ251899). The comparison showed 98% and 82% sequence homology with Rice SAMdC1 and Rice SAMdC2 respectively. The sequence homology indicated that Rice SAMdC cDNA sequence used in the present study seemed to be the SAMdC1 isoform. The Rice SAMdC cDNA sequence obtained from salt-tolerant rice plant (*Oryza sativa* cv. Pokkali) was submitted to GenBank (accession no. AY966487).

Tobacco is a salt sensitive plant and throughout its developmental stage spermidine titre remains high [22]. Hence, further increment of transcript level of SPDS cDNA was not required during cloning from total RNA. The sequence of *Nicotiana tabacum* cv. Jayasri SPDS showed 99% homology with *Nicotiana sylvestris* SPDS[17]. The SPDS sequence obtained from *Nicotiana tabacum* cv. Jayasri was compared with SPDS1 (accession no. AJ251296) and SPDS2 (accession no. AJ251297), isoform sequences of *Arabidopsis thaliana* and 76% and 74% sequence homology were obtained respectively (Multiple sequence alignment, ClustalW). *Nicotiana sylvestris* SPDS cDNA sequence showed 77% sequence homology with *Arabidopsis thaliana* SPDS isoforms SPDS1 and SPDS2. The partial cDNA sequence of *Nicotiana tabacum* cv. Jayasri SPDS was submitted to GenBank (accession no.DQ536198).

Over expression of rSAMdC and tSPDS in non-mutant *E. coli* cells: The rice SAMdC cDNA was expressed as a 'Protein A' tag fusion protein in *E. coli* host strain HB101 using the expression vector pEZZ18. The amino acid sequence, derived from the nucleotide sequence, encoded a polypeptide with 399 amino acid residues and a molecular mass of 43.8 kDa (figure 2A). As revealed by alignment of the derived amino acid sequences (table 2) of various organisms (Multiple sequence alignment, Clustal W), Rice SAMdC cDNA sequence obviously encoded the proenzyme processing site, LSE*SS[23, 24]. The SAMdC pre-protein derived from the cDNA is post translationally modified to form pyruvate containing α and β subunits, which subsequently are joined together by disulfide bond to constitute the functional enzyme[25,26].

The cDNA of Tobacco spermidine synthase was expressed as a GST-tag fusion protein in *E. coli* expression vector pGEX-3X (Amersham Biosciencees), under an IPTG inducible tac promoter. *E. coli* host strain BL21 was used for the overexpression of pGEX-3X:tSPDS. BL21 a *E. coli* host strain was used for the over expression of pGEX-3X:tSPDS. The sequence of amino acids derived from the The amino acid sequence deduced from the Tobacco SPDS nucleotide sequence had 316 amino acids and a molecular mass of 34.7 kDa. The GST: tSPDS fusion proteins of 60 kDa contained 26 kDa glutathionine-S-transferase and 34.7 kDa tobacco SPDS (figure 2B, lane 2).

Purification of rSAMdC and tSPDS proteins: The vector pEZZ18 contained an IgG binding domain i.e. 14 kDa ZZ domain of 'protein A'[19]. Full-length 57.8 kDa (43.8 kDa of rSAMdC+14 kDa of ZZ domain) protein alongwith 23 kDa protein (figure 2B, lane 1, 2, 3) (14 kDa ZZ domain tagged with 9 kDa β subunit of rSAMdC) were obtained by SDS-PAGE analysis (figure 2B, lane 1, 2, 3). Overproduction of recombinant protein might have disturbed the cleavage mechanism and approximately 20% of the full-length protein was produced, as evident from SDS-PAGE (12%) (figure 2B, lane 1, 2, 3). The C-terminal α subunit (35 kDa) was lost during purification through IgG sepharose column. So, the enzymatic assay of rSAMdC was performed with crude recombinant *E. coli* cell lysate. The contribution of endogenous *E. coli* SAMdC enzyme of non-mutant *E. coli* strain was eliminated as negative control (figure 3, figure 4).

Table-2
Alignments of rSAMdC (OsI-SAMdC) sequence with the SAMdC sequences obtained from various organisms and identify the pro-enzyme processing site, the event that occurs during translation of SAMdC transcripts

OsI-SAMdC	44 ALSRAQIDSVLDLARCTIVSELSNKDFDSYV**LSE**	**SS**LFIYSDKIVIKTCGTTKLLLTIPR 103
OsJ- SAMdC	44 ALSRAQIDSVLDLARCTIVSELSNKDFDSYV**LSE**	**SS**LFIYSDKIVIKTCGTTKLLLTIPR 103
Zm- SAMdC	44 ALSRAQIDSVLDLARCTIVSELSNKDFDSYV**LSE**	**SS**LFIYPLKIVIKTCGTTKLLLTIPR 103
Ta- SAMdC	37 ALSRAQIDSVLDLARCTIVSELSTKDFDSYV**LSE**	**SS**LFIYSQKIVIKTCGTTMLLLTIPR 96
Dc- SAMdC	37 TRPQIDSILEPAKCTIVSQLSNKHFDSYV **LSE**	**SS**LFVYPCKMILKTCGTTRLLLSIPVIL96
DC- SAMdC	37 VLSKNQLDEFLGPAECTIVASLSNEHVDSYV**LSE**	**SS**LFVYAYKIIIKTCGTTKLLKSIPP 96
Pn- SAMdC	37 ALSKEQLDKVLKPAECTIVSSLSNNEVDSYV**LSE**	**SS**LFVYPYKIIIKTCGTTKLLLSIPP 96
Nc-SAMdC	39 SLSKAQLDEILGPAECTIVDSLSNDDVDSYV**LSE**	**SS**LFVYSYKIIIKTCGTTKLLLAIPP 98
Ds- SAMdC	39 SLSKAQLDEILGPAECTIVDNLSNDYVDSYV**LSE**	**SS**LFVYSYKIIIKTCGTTKLLLAIPP 98
Cr- SAMdC	37 ALNKSQIDEILEPAECTIVDSLSNQYLDSYV**LSE**	**SS**LFVYPYKIIIKTCGTTKLLLSIPA 96
Gm- SAMdC	37 ALTKSQLGEILTPAACTIVSSLKNDNVDSYV**LSE**	**SS**LFVYAYKIIIKTCGTTKLLLAIPP 96
Vf- SAMdC	35 SLTKSQLDEILAPAECTIVSSLANEDVDSYV**LSE**	**SS**LFVYAYKIIIKTCGTTKLLLAIPP 94
At- SAMdC	35 SLTKSQLDEILTPAECTIVSSLTNSFVDSYV**LSE SS**LFVYPYKIIIKTCGTTKLLLSIPH 94	
Bj- SAMdC4	35 ALTRSQLDEILTPAACEIVSSLSNDHLDSYV**LSE**	**SS**FFVYPYKVIIKTCGTTKLLLSIPP 94
Sp- SAMdC	51 AVSRNDWDDMLAQAQCKVLSVVNSEEIDAYL**LSE**	**SS**MFVFAHKIILKTCGTTTLLASLPR 110
BS- SAMdC	34 TIPRSEWDILLKDVQCSIISVTKTDKQEAYV**LSE**	**SS**MFVSKRRFILKTCGTTLLLKALVP 93
HS- SAMdC	34 TIPRSEWDILLKDVQCSIISVTKTDKQEAYV**LSE**	**SS**MFVSKRRFILKTCGTTLLLKALVP 93
RN- SAMdC	34 TIPRSEWDVLLKDVQCSIISVTKTDKQEAYV**LSE**	**SS**MFVSKRRFILKTCGTTLLLKALVP 93
XL- SAMdC	36 DIPRFEWDKLLENVHCLIISVTKTDKQEAYV**LSE**	**SS**MFVSKRRFILKTCGTTLLLQALVP 95
AP- SAMdC	32 KIPRKKYEDLLKAACCEVISYTSNDKIDAYV**LSE**	**SS**MFVTKRRFILKTCGTTTPIECIKP 91
OV- SAMdC	53 NIPYSELVSMLDIAQCRILHSKSNECMDSYV**LSE**	**SS**MFISDFRIILKTCGTTRLLHAIER 112
TC- SAMdC	52 ALGDEVWKGVVGSLNAQIVSKESNEYIRSYV**LTE**	**SS**LFVMRDRIILITCGTTTLLNAVPF 111

OsI=*Oryza sativa*.Indica, OsJ=*Oryza sativa*.Japunica, Zm=*Zea mays*, Ta=*Triticum aestivum*, Np=*Narcissus pseudonarcissus*, Dc = *Dendrobium crumenatum*, Nc = *Nicotiana tabacum*, Cr = *Catharanthus roseus*, Pn = *Pharbitis nil*, Ds = *Datura stramonium*, Vf = *Vicia faba*, Gm = *Glycine max*, At = *Arabidopsis thaliana*, DC = *Daucus carota*, Bj = *Brassica juncea*, Sp = *Schizosaccharomyces pombe*, BS = *Bos taurus*, OV = *Onchocerca volvulus*, XL = *Xenopus laevis*, DM = *Drosophila melanogaster*, TC = *Trypanosoma cruzi*, AP = *Acyrthosiphon pisum*, RN = *Rattus norvegicus*, HS = *Homo sapiens*.

Tobacco SPDS was expressed as a GST-tag fusion protein in *E. coli* expression vector pGEX-3X (Amersham Biosciences), containing IPTG-inducible tac promoter. Purified GST:tSPDS fusion protein, eluted from the glutathione-sepharose column, was checked by SDS-PAGE (12%) (figure 2B, lane 2). The fusion protein (pGEX-3X:tSPDS) was cleaved with factor Xa to get pure tSPDS protein and GST (figure 2B, lane 3).

Characterisation of rSAMdC and tSPDS proteins: Rice SAMdC protein had a short half-life as it contains a putative PEST sequence[26], so the cell lysate of recombinant *E. coli* cells, HB101:pEZZ18: rSAMdC (figure 3), was used for characterization of the expressed rSAMdC. For characterization of tSPDS, the cell lysate of BL21: pGEX-3X: tSPDS (figure 3) were used. The cell lysate of HB101:pEZZ18 and BL21: pGEX-3X was used as control (figure 3). Increment of spermidine production was noticed when HB101:pEZZ18 and BL21: pGEX-3X cell lysates were supplemented with exogenous SAM and Putrescine (figure 3). Spermidine production was not increased considerably in HB101:pEZZ18:rSAMdC or BL21:pGEX-3X:tSPDS cell lysates (figure 3) until supplemented with exogenous SAM (figure 3) or SAM together with Putrescine (figure 3). Addition of purified tSPDS protein in HB101:pEZZ18: rSAMdC cell lysate alone or with exogenous SAM showed increased spermidine production (figure 3). The spermidine level enhanced remarkably when purified tSPDS protein along with exogenous SAM and Putrescine were added to the cell lysate of HB101: pEZZ: rSAMdC (figure 3).

Complementation assays: In *E. coli* complementation assay is an established powerful tool to study the activity of the enzymes[28]. Complementation of polyamine-deficient *E. coli* mutant by heterologous expression of mouse ornithine decarboxylase could reconstitute the polyamine biosynthetic pathway, as evident from the assay of ODC activity [29]. *E. coli* host strain HT551 (*71.18Δ Spe ED*), deficient in most of the SPDS and SAMdC genes, was used for complementation assays with *Nicotiana sylvestris* spermidine synthase gene[17]. *E. coli* mutant HT252[30] containing deletion mutation in the genes Spe A (Arginine decarboxylase), Spe B (Agmatinureohydrolase), Spe C (Ornithine decarboxylase) Spe ED (Spermidine synthase, Adenosylmethionine decarboxylase) was used as a host strain in the present study. Expression vectors containing rSAMdC and tSPDS were separately transformed in HT252. Cell lysate of recombinant HT252 strains were prepared[31] for functional assays of the respective enzymes.

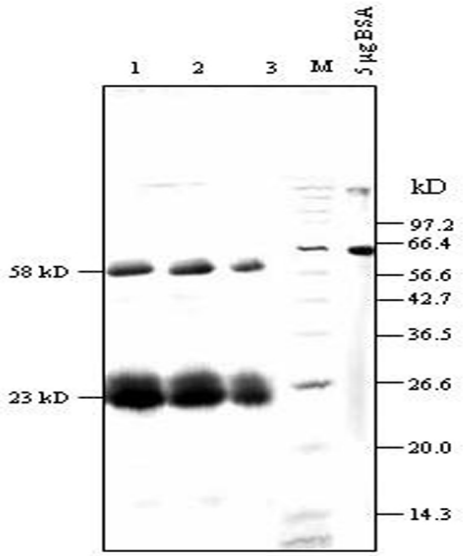

Figure2A

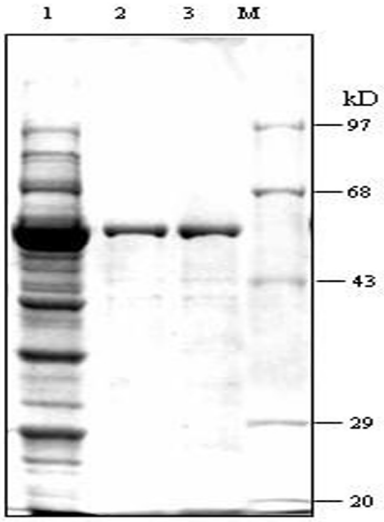

Figure2B

Figure-2

(A) Overexpression of rSAMdC in *E. coli* expression vector pEZZ18. 57.8kDa (43.8kDa of rSAMdC+ 14 kDa of zz domain) of rSAMdC (pre-protein) over expressed as full length in pEZZ18 vector was purified by IgG sepharose column and was checked by running 12 % PAGE (SDS^+). Lane 1, Lane 2 and Lane 3, purified fraction of PEZZ:rSAMdC; Lane M, Mol.Wt marker. (B) Overexpression of tSPDS in *E. coli* expression vector pGEX-3X and purification of the recombinant protein using glutathione sepharose affinity resin. SDS PAGE analysis of the recombinant GST:tSPDS protein. Lane 1, aliquot of the protein extract (S_{10}) from the *E. coli* BL21:pGEX-3X:tSPDS; Lanes2, Purified fraction of GST:tSPDS (26 kDa + 34 kDa= 60 kDa); Lanes 3, GST:tSPDS cleaved with factor Xa to get pure tSPDS protein and GST); Lane M, Molecular weight marker

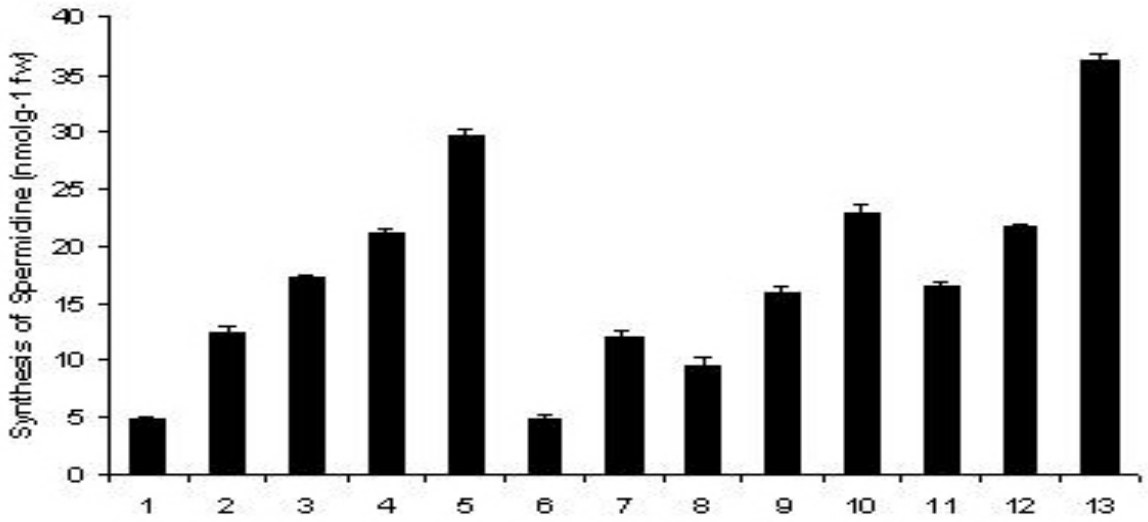

Figure-3

Characterisation of rSAMdC and tSPDS proteins: The histogram is representing the production levels of spermidine in different experimental conditions. Bars 1 – 13: HB101:pEZZ18; HB101:pEZZ18 + SAM + Putrescine; HB101:pEZZ18:rSAMdC; HB101:pEZZ18:rSAMdC + SAM; B101:pEZZ18:rSAMdC + SAM + Putrescine; BL21:pGEX-3X; BL21:pGEX-3X + SAM + Putrescine; BL21:pGEX-3X:tSPDS; BL21:pGEX-3X:tSPDS + SAM; BL21:pGEX-3X:tSPDS + SAM + Putrescine; HB101:pEZZ18:rSAMdC + tSPDS; HB101:pEZZ18:rSAMdC + tSPDS + SAM; HB101:pEZZ18:rSAMdC + tSPDS + SAM + Putrescine.

In presence of SAM and Putrescine, cell lysate of recombinant HT252 expressing rSAMdC was taken alone or was added with the cell lysate of recombinant HT252 expressing tSPDS, to reconstitute the polyamine biosynthetic pathway in *E. coli*. Production of spermidine was measured to show the activity of the enzymes. For SPDS, both the cell lysate of recombinant HT252 containing tSPDS and the purified tSPDS enzyme were used. Maximum spermidine production was noticed when both rSAMdC & tSPDS enzymes (in cell lysates / pure form) were involved in utilizing Putrescine and SAM (figure 4). Thus the reconstitution of the *E. coli* polyamine biosynthetic pathway by the functional activity of two heterologous enzymes was evident.

Mutant complementation experiment: Reconstitution of the polyamine biosynthetic pathway in *E. coli* mutant HT252 by rSAMdC and tSPDS has been done by mutant complementation experiment. Cell lysates of HT252 showed the presence of trace amount of spermidine when supplemented with SAM or Putrescine or both (figure 4). The cell lysate of HT252 containing pEZZ18: rSAMdC showed considerable amount of spermidine, when supplemented with SAM, Putrescine or both (Figure 4). Recombinant HT252, containing pGEX-3X: tSPDS, on the other hand, showed very low amount of spermidine, in the absence of SAM or Putrescine (figure 4). It may be postulated that in the absence of rSAMdC, SAM and Putrescine, the overproduction of tSPDS alone is not sufficient to synthesize high amount of spermidine. A sharp increase of spermidine production was noticed, when HT252: pGEX-3X: tSPDS was supplemented with SAM, Putrescine or both (figure 4). When cell lysates of HT252:pEZZ18: rSAMdC and HT252: pGEX-3X: tSPDS were taken together and SAM and Putrescine were added exogenously, spermidine production increased remarkably (figure 4).

Effect of purified tSPDS protein on the production of spermidine, was studied in the presence of cell lysate of HT252:pEZZ18: rSAMdC, SAM and Putrescine. Maximum enhancement of spermidine production with the addition of purified tSPDS protein was noticed (figure 4).

Exposure of high salt (100-150 mM NaCl) has resulted in significant increase of spermidine and spermine level in a number of plants[5]. Furthermore, elevation of concentration of spermidine and spermine was found to be induced by the exposure of NaCl in wheat root though putrescine concentration remained unaltered[10]. Capell et al[29] reported that conversion of putrescine to spermidine and spermine was important to confer drought tolerance in plants.

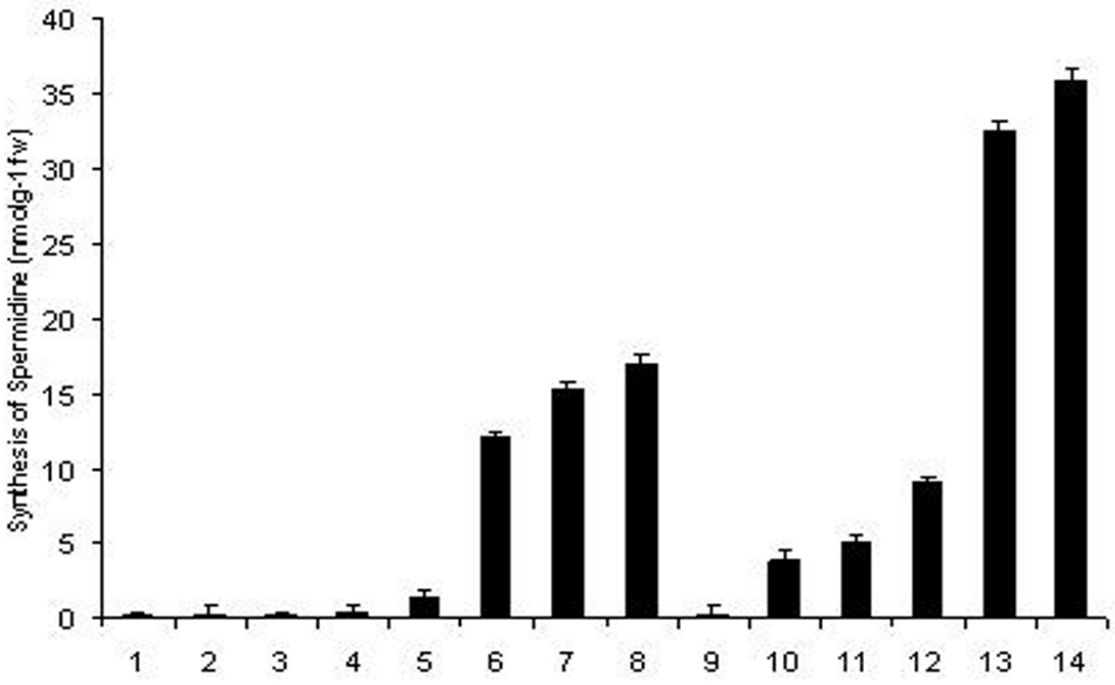

Figure-4

Characterisation of rSAMdC and tSPDS proteins by mutant complementation experiment: The histogram is representing the production levels of spermidine in different experimental conditions. Bars 1-14: HT252; HT252 + SAM; HT252 + Putrescine; HT252 + SAM + Putrescine; HT252:rSAMdC; HT252:rSAMdC + SAM; HT252:rSAMdC + Putrescine; HT252:rSAMdC + SAM + Putrescine; HT252:tSPDS; HT252:tSPDS + SAM; HT252:tSPDS + Putrescine; HT252:tSPDS + SAM + Putrescine; HT252:tSPDS + HT252:rSAMdC + SAM + Putrescine; HT252:rSAMdC + tSPDS + SAM + Putrescine

Conclusion

In the present study, a polyamine deficient strain of *E.coli* HT252 has been used. The mutant strain lacks polyamine biosynthetic enzymes. Spermidine production occurs by a coupled reaction involving both SAMdC and SPDS. Hence, the activity of both the enzymes can be assayed by measuring the spermidine production. In this context we have designed an *in vitro* reconstitution assay for these two enzymes simultaneously. Furthermore, the present study has provided clear evidence that: i. one or more than one heterologous enzyme / enzymes can reconstitute the polyamine biosynthetic pathway successfully in mutant *E.coli*, and ii. effective enhancement of spermidine production requires active participation of SAMdC and SPDS simultaneously. To design new strategies of plant survival in the changing environment, the gain of function study involving both the enzymes along with the experimental planning has high significance.

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