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Differential role of spermine and thermospermine in *Arabidopsis thaliana* in response to abiotic stresses

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Abstract

Polyamines (PA) are small organic polycations found in all living organisms. Putrescine (Put), spermidine (Spd), spermine (Spm) and thermospermine (T-Spm) are the most abundant PAs in plants. Of the polyamines (PAs) plant contains at least two types of tetraamines; spermine (Spm) and thermospermine (T-Spm) and they seem to have different functions in plants. The experiment was conducted to elucidate the stress responsive roles of Spm and T-Spm using wild ecotype (Col-0) and three different knockout mutants (*spms*, *acl5* and *spms/acl5*) of *Arabidopsis*, differing in the amount of spermine and thermospermine content. First exogenous pretreatment of both tetraamines showed their different effects on plant phenotype including root and shoot length, chlorophyll content both under normal and salt stressed condition. The effect of exogenous T-Spm was more effective than Spm in plant growth and stress tolerance. The mutants varying in endogenous tetraamines content were also found to be different in response to stress (salt, osmotic and ionic) condition. Growth inhibition of *spms* mutant seedlings was minimum in compare to other mutants and wild type, probably due to higher amount of T-Spm content. Further to differentiate the tissue specific expression of tetraamines, histochemical GUS assay was performed using *SPMS* and *ACL5* promoter:: GUS transgenic plants and the results indicate that the two tetraamines; spermine and thermospermine may play some different and specific role in respect to tissue specificity and stress responses.

Introduction

Polyamines (PA) are positively charged low molecular weight aliphatic molecule having two or more amino groups, found in almost all living organisms (Kusano *et al.*, 2007). Due to polycationic nature, they have high affinity to different anionic cellular components such as DNA, RNA and protein. Major polyamines in plants are diamine putrescine (Put), triamine spermidine (Spd), tetraamine spermine (Spm) and thermospermine (T-Spm). (Kusano *et al.*, 2008; Fuell *et al.*, 2010; Tiburcio *et al.*, 2014). Polyamine plays major role in plant growth, development as well as cell division. They are involved not only in fundamental cellular processes but also in various environmental stresses (Alcazar *et al.*, 2006; Bagni *et al.*, 2006; Kusano *et al.*, 2008).

In plants, PA biosynthesis starts with the formation of Put, which is synthesized from ornithine by ornithine decarboxylase. Putrescine is also synthesized from arginine via agmatine by three sequential reactions catalyzed by arginine decarboxylase (ADC), agmatine iminohydrolase (AIH), and *N*-carbamoylputrescine aminohydrolase (CPA), respectively. Then, Put is converted to Spd and Spm via two sequential aminopropyl transferase reactions catalyzed by Spd synthase (SPDS) and Spm synthase (SPMS), respectively. In both cases, an aminopropyl residue is transferred from the decarboxylated S-adenosylmethionine which is synthesized by S-adenosylmethionine decarboxylase (SAMDC) (see review, Kusano, *et al.*, 2008). The genome of the model plant *Arabidopsis thaliana* does not contain an ornithine

decarboxylase (ODC) gene (Hanfrey *et al.*, 2001). In this organism, it had been believed that two genes, *Acaulis 5* (*ACL5*) and *Spermine synthase* (*SPMS*), encode SPMS. However, Knott *et al.* (2007) reported that the bacterially expressed recombinant ACL5 protein catalyzes the conversion of Spd to T-Spm, but not to Spm, indicating that *ACL5* encodes T-Spm synthase. The above discussion indicates that *Arabidopsis* plants contain four major PAs including T-Spm. T-Spm was first discovered in the thermophilic bacterium *Thermus thermophilus* (Oshima, 1979) and then found in several plants including *Pisum sativum*, *Vicia sativa* and *Malus sylvestris* (Hamana *et al.*, 1991; Kitashiba *et al.*, 2005). Recently, Naka *et al.* (2009) detected T-Spm in *Nicotiana tabacum*, *Lycopersicon esculentum* and *Oryza sativa*. The gathered evidence suggests that T-Spm seems to be ubiquitously present in plants. As all the genes involved in PA biosynthetic pathway identified in *A. thaliana* and thus the genetic analyses of the loss/gain-of-function types became possible. With such analyses, it has been revealed that Put and Spd are essential for normal growth of *Arabidopsis*, while Spm and T-Spm are not (Alcazar *et al.*, 2006). *Arabidopsis* (*spms/acl5*) mutant plant that cannot produce spermine, was hypersensitive to drought and salinity and the hypersensitive phenotype was mitigated by application of exogenous spermine and the same mutant also showed a phenotype resembling Ca^{2+} -deficiency (Yamaguchi *et al.*, 2006). *Arabidopsis* plants overexpressing *Cucurbita ficifolia* Spd synthase gene became tolerant to multiple stresses such as chilling,

freezing, salinity, drought and paraquat-induced oxidative stress (Kasukabe *et al.*, 2004). Similarly, several other plant species also shown that polyamine accumulation occurs in response to several adverse environmental conditions, including salinity, drought, chilling, heat, hypoxia, ozone, UV-B and UV-C, heavy metal toxicity, mechanical wounding and herbicide treatment (Alcázar *et al.*, 2006; Groppa *et al.*, 2008). Spm, one of the tetraamines, has a signalling function to induce a defense reaction against avirulent viral pathogen. Similar evidence was obtained in the combination of *A. thaliana* carrying the leucine rich repeat type resistant gene *RCY1* and the yellow strain of cucumber mosaic virus (CMV) (Mitsuya *et al.*, 2009). Recently, proven that another tetraamine, T-Spm also has an inducing activity of the expression of genes involving in the same Spm-signal pathway and of the genes involving in CMV-triggered hypersensitive response (HR) and consistent with the gene-inducing activity, T-Spm can also represses the CMV multiplication as efficiently as Spm does (Sagor *et al.*, 2012). In this study, we examined the roles of both exogenous and endogenous Spm and T-Spm in response to abiotic stresses and also their tissue specific expression in *Arabidopsis thaliana*.

Materials and Methods

Experimental site, plant materials and growth condition

The experiment was conducted at the Tissue Culture Laboratory and Plant Molecular Genetics Laboratory, Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh. The plant materials consists of three different *Arabidopsis* mutants namely *spms*, *acl5* and *spms/acl5* including wild type Col-0. The list of the plant materials and their characteristics has been shown in Table 1. Seeds of all mutants and wild type were surface sterilized with 70% ethanol for 1 min and 1% sodium hypochloride plus 0.1% Tween-20 for 15 min, followed by extensive washing with sterile distilled water. After sterilization seeds were placed on half-strength MS (Murashige and Skoog, 1962) 1.5% agar plates (pH 5.6) containing 1% sucrose and B5 vitamin (MP Biomedicals, Cat#2625149). The plants were grown at 22 °C under a 14h light /10h dark photoperiod.

Table 1. Plant materials, characteristics and source of the material

Plant Material	Special characteristics	Source
Col-0	Polyamines are in normal level	Sagor <i>et al.</i> , 2013
<i>spms</i>	No Spm, slightly increased level of T-Spm	Sagor <i>et al.</i> , 2013
<i>acl5</i>	No T-Spm, slightly increased level of Spm	Sagor <i>et al.</i> , 2013
<i>spms/acl5</i>	No Spm and T-Spm	Sagor <i>et al.</i> , 2013
<i>SPMS</i>	Polyamines are in normal level	Sagor <i>et al.</i> , 2013
pro::GUS		
<i>ACL5</i>	Polyamines are in normal level	Sagor <i>et al.</i> , 2013
pro::GUS		

Growth response to stress condition

Sterilized *Arabidopsis* seeds were grown on 1/2 MS agar plates containing different concentration of NaCl (0, 25, 50, 100 mM); Polyethylene glycol (PEG-6000) (0%, 10%, 15%, 20%); CaCl₂ (0, 6, 12, 50 mM) and KCl (0, 50 mM). The plates were placed at a vertical position with an 85 degree angle and incubated at 22°C for 14 days. NaCl, PEG, CaCl₂ and KCl were used to induce salt, osmotic and ionic stress, respectively.

Pretreatment with Spm and T-Spm solution

Ten days old seedlings of Col-0 were carefully detached from the MS agar plate, rinsed with distilled water, placed the root in wet filter paper containing 500 µM Spm and 100 µM T-Spm solution for 24 hrs. Then, the plants were carefully transferred to 0, 100 and 200 mM NaCl containing MS media and shifted to tissue culture room at 22°C.

Data collection and analysis

Root length (cm): Length of root was measured in centimeter (cm) from the base to the tip of the roots by a measuring scale at 14 days after initiation.

Shoot length (cm): The shoot length was measured with the help of centimeter scale by placing the plants straightly on glass slide from the starting part of main stem to tip of the upper leaf.

Leaf area: Leaf area was measured under digital optical microscope (Kenis).

Leaf Chlorophyll content [CC] (SPAD unit): Chlorophyll content was measured from one leaves per replication using the SPAD (Minolta-502).

Each experiment was repeated three times and the mean values of collected numerical data were analyzed using Microsoft Excel-2007 software.

Histochemical GUS assay

The histochemical localization of GUS activity was assayed according to the method of Jefferson, 1987; Sagor *et al.*, 2011). Samples were collected and incubated with GUS staining solution [0.5 mg/ml X-Glu (5-bromo-4-chloro-3-indolyl b-D-glucuronide), 10 mM phosphate buffer, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100, 10 mM EDTA] in the dark at 37 °C for overnight. After incubation, stained plant cells were cleared by 70% ethanol to remove the chlorophyll. Samples were taken on light plate and photographed using digital camera (Canon R250, Japan).

Chemicals

Spm were purchased from Tokyo Kasei Co. Ltd. (Tokyo, Japan). T-Spm was chemically synthesized (Niitsu and Samejima, 1986). All other chemicals were obtained at the analytical grade from Sigma-Aldrich Corp (St. Louis, MO, USA), Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Nacalai-Tesque Ltd. (Kyoto, Japan).

Results

Effect of exogenous tetraamines in response to salt stress

In order to notice the effect of exogenous tetraamines, the wild type plants were grown on normal MS media, treated with water (Mock), 500 μ M Spm and 100 μ M T-

Spm for 24 hrs and then transferred to with or without NaCl containing media. Seven days after incubation, T-Spm pretreated plant showed higher root length, shoot length and chlorophyll content compare to Spm and mock one (Fig. 1A, 1B, 1C) both in control and salt stressed conditions.

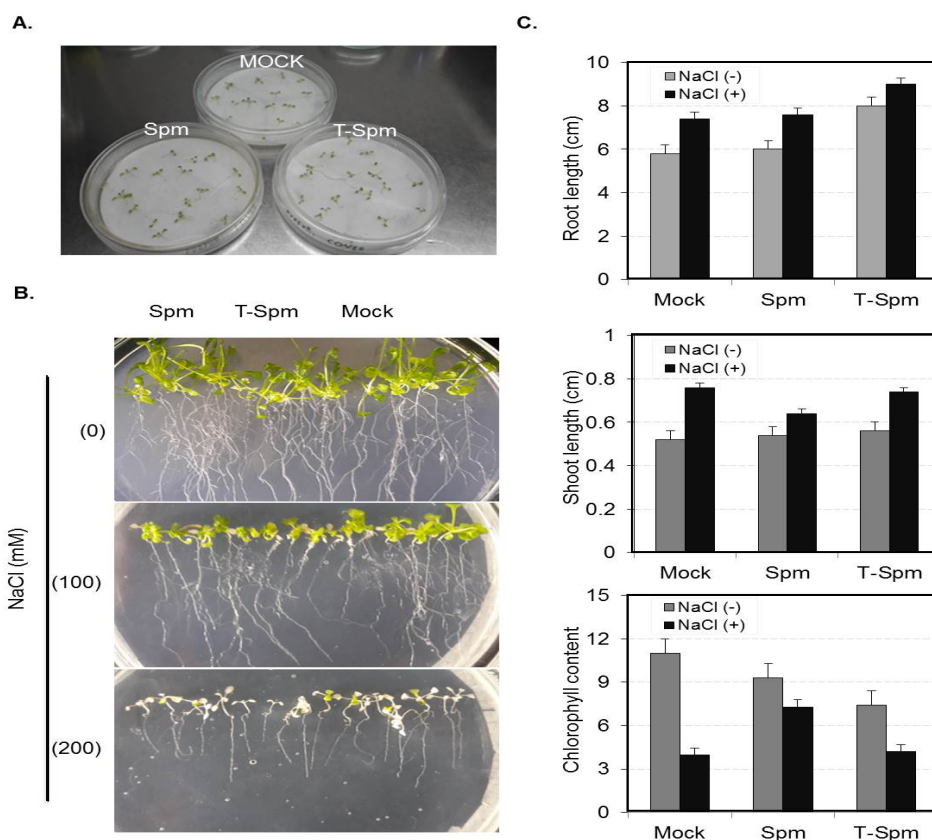


Fig. 1. Effect of exogenously applied Spm and T-Spm to protect *Arabidopsis* seedlings from salt induced damage. A. Pretreatment of ten days old *Arabidopsis* seedlings with 0.5 mM Spm and 0.1 mM T-Spm for 24 hrs. B. Pretreated plants were transferred to 0, 100 and 200 mM NaCl containing MS media and incubated at 22°C for another 5 days and the photograph was taken. C. Root length, shoot length and chlorophyll content in wild type and different tetraamine mutants. Mean values \pm SE were shown using three time repetitions.

Sensitivity of tetraamines deficient mutant to salt stress

The growth response of three mutants namely *spms*, *acl5*, *acl5/spms* and wild type (Col-0) of *Arabidopsis thaliana* were tested under both control and salt stress condition created by using different concentration of NaCl (0, 25, 50 and 100 mM NaCl respectively). Both the wild type and mutant plants were tall, healthy, and vigorous with juvenile leaves, petiole and stems as well as well-established root system at normal physiological condition, but in case of salt stress condition Col-0 showed higher sensitivity whereas *spms* showed the tolerance (Fig. 2). The root length and shoot length were decreased with the increasing concentration of NaCl. At strong salt stress condition, root and shoot length were maximum in *spms* mutant and minimum in *spms/acl5* mutant (Fig. 2; Table 2). Leaf area and total chlorophyll content also showed similar results that *spms* performed the best among the wild type and mutant plants (Table 2).

Growth response of *Arabidopsis* to osmotic stress

Next, the growth response of wild type and *spms*, *acl5*, *spms/acl5* mutants of *Arabidopsis thaliana* were tested under osmotic stress condition created by using PEG solution. The wild type and mutant lines (*spms*, *acl5*, *acl5/spms*) showed varying levels of tolerance under osmotic stress. The growth of plants becomes severely affected at 20% PEG (Fig. 3). Leaves became small and curly, petiole became short and curved, and plant height became also reduced with the increase of PEG level (Fig. 3). Col-0 was highly sensitive to PEG at 20% level as root length and shoot length were very short and *spms* plants were tolerant (Table 3). Other mutant plants showed similar type of sensitivity (Fig. 3). Total leaf area and chlorophyll content were also found to be maximum in *spms* mutant whereas minimum in *spms/acl5* plants at high level of osmotic (20%) stress (Table 3).

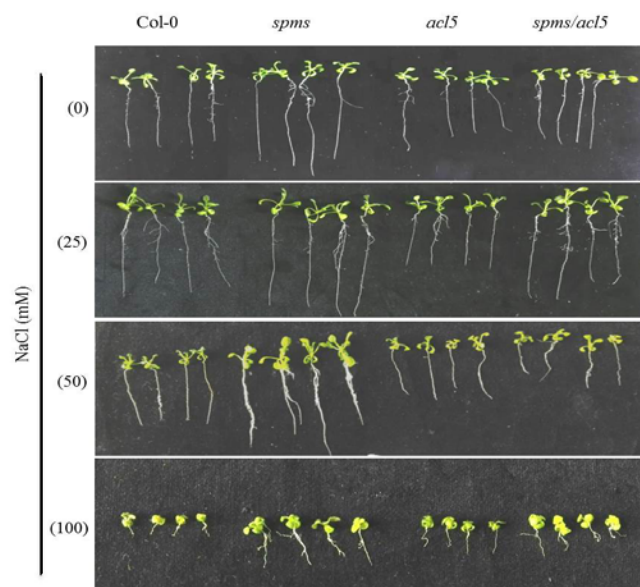


Fig. 2. Growth response of wild type and tetraamine deficient mutant in response to salt stress. Seeds of wild type, *spms*, *ac15* and *spms/ac15* mutants were sown on 0, 25, 50 and 100 mM NaCl containing MS media, then kept on 4°C for two days and then transferred to growth room at 22°C. Photographs were taken after 14 days of incubation

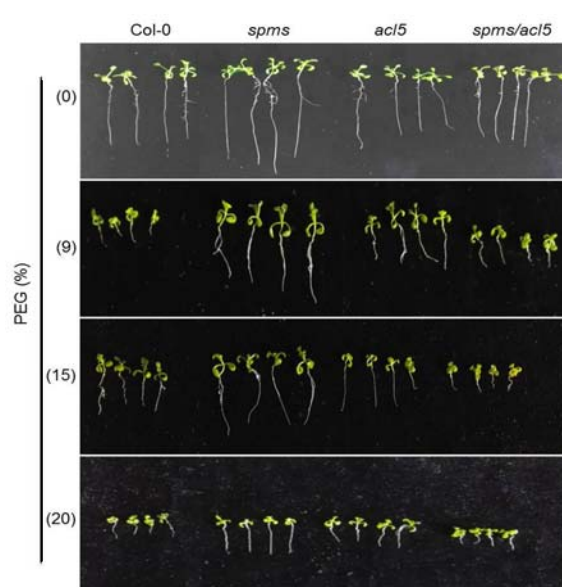


Fig. 3. Phenotypic performance of wild type and tetraamine deficient mutant to osmotic stress. Seeds of wild type, *spms*, *ac15* and *spms/ac15* mutants were sown on 0%, 10%, 15% and 20% PEG containing MS media, then kept on 4°C for two days and then transferred to growth room at 22°C. Photographs were taken after 14 days of incubation

Table 2. Phenotypic characterization of *Col-0*, *spms*, *ac15* and *spms/ac15* in response to salt stress

Plant materials	NaCl (mM)															
	RL (cm)				SL (cm)				LA (mm ²)				CC (unit)			
	0	25	50	100	0	25	50	100	0	25	50	100	0	25	50	100
Col-0	2.3	2.7	2.1	0.6	1.2	1.0	0.4	0.1	6.0	5.4	4.1	2.5	5.0	4.1	2.9	0.6
<i>spms</i>	3.6	3.6	3.0	1.2	1.5	1.1	0.5	0.2	6.5	5.8	4.5	3.2	6.9	4.7	3.5	0.9
<i>ac15</i>	2.6	2.4	1.9	0.4	1.0	0.7	0.2	0.1	5.5	5.0	3.7	2.5	5.2	3.8	2.6	0.4
<i>spms/ac15</i>	2.7	2.2	1.7	0.6	1.0	0.6	0.2	0.1	5.4	5.4	3.6	2.6	5.4	3.1	2.6	0.3

Foot note: Root length (RL); Shoot length (SL); Leaf area (LA) and Chlorophyll content (CC)

Table 3. Phenotypic characterization of *Col-0*, *spms*, *ac15* and *spms/ac15* in response to osmotic stress

Plant materials	NaCl (mM)															
	RL (cm)				SL (cm)				LA (mm ²)				CC (unit)			
	0	10	15	20	0	10	15	20	0	10	15	20	0	10	15	20
Col-0	2.3	1.1	1.0	0.4	1.1	0.8	0.3	0.2	6.0	2.5	2.5	1.8	5.6	2.5	2.1	1.9
<i>spms</i>	3.5	1.5	1.3	0.8	1.5	1.2	0.6	0.3	6.5	3.8	3.6	2.3	7.0	3.1	2.9	2.0
<i>ac15</i>	2.6	0.3	0.6	0.4	1.0	0.7	0.2	0.1	5.6	3.0	2.9	2.2	5.2	2.1	1.9	0.9
<i>spms/ac15</i>	2.7	0.2	0.5	0.3	1.0	0.5	0.3	0.1	5.4	2.3	2.1	1.9	5.4	2.1	1.7	0.7

*Root length (RL); Shoot length (SL); Leaf area (LA); and Chlorophyll content (CC)

Effect of ionic imbalance on the growth response of *Arabidopsis*

Finally the role of tetraamines in response to ionic imbalance condition was evaluated using wild type and tetraamine deficient mutants using CaCl₂ and KCl as inducers. The growth of all the plants was inhibited but

the rate of decrease was minimum in *spms* mutant whereas other three showed almost same degree and level (Fig. 4). Root and shoot length, leaf area, chlorophyll content all were maximum in *spms* mutant compared to others indicating tolerance to ionic imbalance conditions (Fig. 4; Table 4).

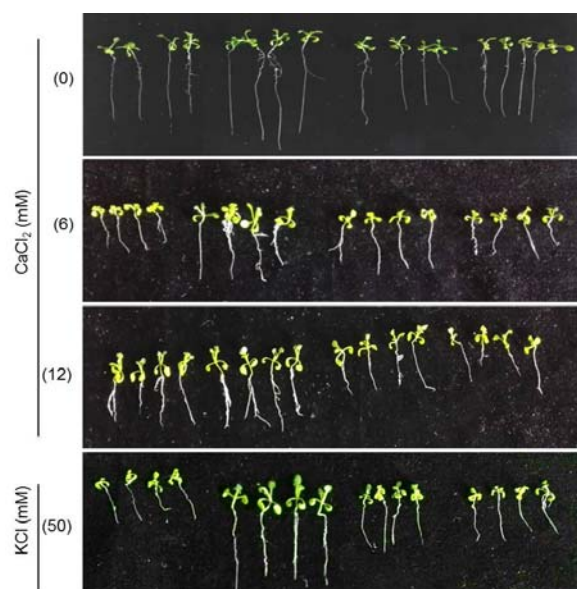


Fig. 4. Effect of ionic imbalance on growth response of *Arabidopsis thaliana*. Seeds of wild type, *spms*, *acl5* and *spms/acl5* mutants were sown on 6 and 12 mM CaCl_2 and 50 mM KCl containing MS media, were kept on 4°C for two days and then transferred to growth room at 22°C. Photographs were taken after 14 days of incubation

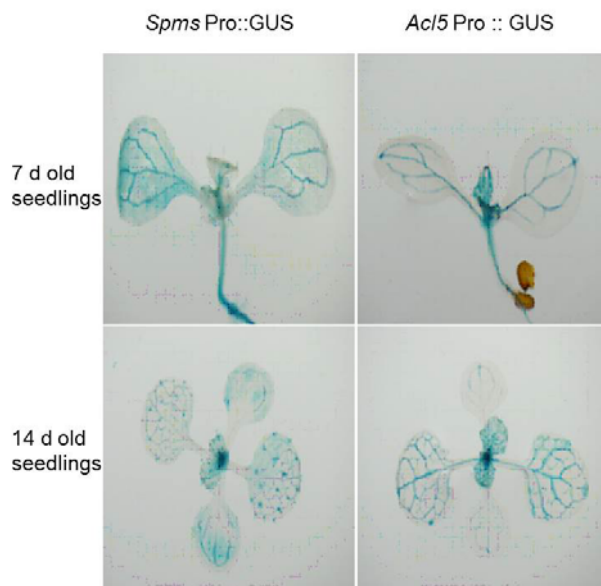


Fig. 5. Expression patterns of *Spms*- and *Acl5*-promoter driven beta-glucuronidase reporter gene (*GUS*) in transgenic plants. Degree of blue staining reflects the promoter activity

Table 4. Phenotypic characterization of Col-0, *spms*, *acl5* and *spms/acl5* in response to ionic stress

Plant materials	CaCl_2 (mM)												KCl (mM)							
	RL (cm)			SL (cm)			LA (mm^2)			CC (unit)			RL (cm)		SL (cm)		LA (mm^2)		CC (unit)	
	0	6	12	0	6	12	0	6	12	0	6	12	0	50	0	50	0	50	0	50
Col-0	2.3	1.0	1.2	1.2	0.5	0.3	6.0	2.3	2.2	5.6	2.1	1.3	2.3	0.6	1.2	0.1	6.0	1.9	5.6	0.9
<i>spms</i>	3.5	1.3	1.5	1.5	0.8	0.5	6.5	3.2	2.9	6.9	2.9	2.1	3.5	0.8	1.5	0.2	6.5	2.5	6.9	1.3
<i>acl5</i>	2.6	0.6	0.5	0.9	0.4	0.2	5.6	2.2	1.6	5.2	2.0	1.4	2.6	0.3	0.9	0.1	5.6	1.9	5.2	0.5
<i>spms/acl5</i>	2.7	0.4	0.4	0.8	0.4	0.2	5.4	1.9	1.8	5.4	1.7	1.2	2.7	0.2	0.8	0.1	5.4	1.8	5.4	0.4

*Root length (RL); Shoot length (SL); Leaf area (LA); and Chlorophyll content (CC)

Expression of Spermine synthase (SPMS) and Thermospermine synthase (ACL5):

To investigate the normal expression pattern of spermine synthase (*Spms*) and thermospermine synthase (*Acl5*) genes, transgenic plants namely *Spms* promoter::GUS and *Acl5* promoter::GUS were grown on normal MS media for seven and fourteen days. After that the plants were carefully detached from the media and subjected to undergo GUS staining according to Sagor *et al.* (2011). Both the *Spms* and *Acl5* promoter activity was detected in the cotyledons, true leaves and roots, but main difference is the strong induction of *Acl5* in the vascular parts of the plants indicating some specific role in xylem differentiation and vascular development.

Discussion

Polyamines are positively charged aliphatic molecule with low molecular weight having two or more amino group which is found in all living organisms. The present study was planned to reveal the differential roles of tetraamine (Spm and T-Spm) in *Arabidopsis thaliana* using wild type (Col-0) and mutant lines (*spms*, *acl5*, *spms/acl5*) having different concentrations of spermine and thermospermine (Sagor *et al.*, 2013). At first effect of exogenous spermine and thermospermine on wild

type was examined under both normal and salt stress condition. T-Spm pretreated plants showed the highest root and shoot length compared to mock or Spm treated one. The chlorophyll content was also found to be higher in T-Spm pretreated plant. Similar results were also found by Knott *et al.*, (2007), Minguet *et al.*, (2008), Takano *et al.*, (2012), where they showed that exogenous T-Spm can also act as hormone for the growth and development of plant via activating auxin or cytokinin signaling pathway gene and also hormonal deregulation. The wild type and both tetraamine deficient mutant (*spms/acl5*) or individual (either *spms* or *acl5*) mutants were grown on normal MS media or under stressful conditions but there was not much specific difference among wild type and mutant lines (*spms*, *acl5* and *spms/acl5*) at seedling stages. Kakehi *et al.* (2008) showed *acl5* mutant had dwarf phenotype at mature stage due to lack of T-Spm, but *spms* mutant had not such difference. In case of stress condition *spms* showed the highest tolerance than other wild and mutants through investigation of different parameter (root length, shoot length, leaf area and chlorophyll content). Yamaguchi *et al.* (2006, 2007) showed that tetraamine deficient mutant can compensate salt tolerance phenotype by exogenous application of Spm

which was little bit different from our findings, but this may be due to some differential and tissue specific role of two different tetraamines; Spm and T-Spm. *spms* mutant cannot produce Spm but slightly higher T-Spm compared to normal plant (Sagor et al., 2011; Naka et al., 2009). This increase of T-Spm may have some crucial role in stress response. The expression pattern of tetraamine synthase genes were also studied using promoter::GUS transgenic approach. Both the *SPMS* and *ACL5* gene differ from each other in terms of intensity and expression site. The intensity of *ACL5* gene expression was found only in vascular system, whereas, *Spms* was throughout the leaf and stem including vascular part. Similar results were also obtained by Sagor, et al., (2011), Kakehi et al., (2008). They also found that *ACL5* is highly involved in xylem differentiation and vascular development, whereas *Spms* is in stress responses (Clay and Nelson, 2005). All of the above discussion suggested that in *Arabidopsis* both the tetraamines have some specific and differential role based on their amount present either in soluble or conjugated form.

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