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Norspermine substitutes for thermospermine in the control of stem elongation in *Arabidopsis thaliana*

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ABSTRACT

Thermospermine is a structural isomer of spermine and is required for stem elongation in *Arabidopsis thaliana*. We noted the C3C3 arrangement of carbon chains in thermospermine (C3C3C4), which is not present in spermine (C3C4C3), and examined if it is functionally replaced with norspermine (C3C3C3) or not. Exogenous application of norspermine to *acl5*, a mutant defective in the synthesis of thermospermine, partially suppressed its dwarf phenotype, and down-regulated the level of the *acl5* transcript which is much higher than that of the *ACL5* transcript in the wild type. Furthermore, in the *Zinnia* culture, differentiation of mesophyll cells into tracheary elements was blocked by thermospermine and norspermine but not by spermine. Our results indicate that norspermine can functionally substitute for thermospermine.

1. Introduction

Polyamines are small cationic molecules with two or more primary amino groups present in all living cells and play important roles in cell growth and development [1,2]. Among the three major polyamines (putrescine, spermidine, and spermine), spermidine and spermine are formed successively from putrescine through the addition of an aminopropyl moiety of decarboxylated *S*-adenosyl methionine (dcSAM) by each specific aminopropyl transferase (APT), spermidine synthase (SPDS) and spermine synthase, respectively. Spermidine is a substrate for the hypusine modification of the eukaryotic translation initiation factor 5A (eIF5A), which is essential in all eukaryotic cells [3]. Due to their cationic nature, polyamines, in particular spermine, have high binding affinity to RNA, DNA, proteins, and other acidic substances, and participate in many cellular processes. One of the major functions of intracellular polyamines may be in regulating mRNA translation because most polyamines exist in polyamine–RNA complex within cells [4]. Polyamines are also proposed to be a source of hydrogen peroxide, which is produced through polyamine oxidative degradation, and play a role in stress responses. In higher plants, polyamines are accumulated extracellularly in response to pathogen attacks and their degradation by apoplastic polyamine oxidase (PAO) results in the production of hydrogen peroxide, which may, in turn, activate defense pathways against pathogens [5,6].

Plant cells also contain thermospermine, a structural isomer of spermine, which was first detected in an extreme thermophile, *Thermus thermophilus* [7]. Thermospermine is formed from spermidine by the action of thermospermine synthase, an enzyme similar to spermine synthase [8]. Phylogenetic analyses, however, suggest that thermospermine synthase was acquired by an algal ancestor of plants through horizontal gene transfer from archaea while spermine synthase evolved from SPDS in respective lineages of plants, animals, fungi, and bacteria [9]. Although thermospermine has been sporadically found in animal systems [10], its function remains unknown. We have shown that the *acaulis5* (*acl5*) mutant of *Arabidopsis thaliana*, which exhibits severe dwarfism with increased vein thickness and vascularization in stems [11,12], is defective in the synthesis of thermospermine and exogenously-applied thermospermine partially restores the mutant phenotype [13]. A study of the *thickvein* (*tkv*) mutant, an allele of *ACL5*, suggests that the boundary between veins and non-vein regions is defined by *ACL5/TKV* whose expression is specific to provascular cells

Abbreviations: ACL5, *ACAULIS5*; APT, aminopropyl transferase; PAO, polyamine oxidase; *PHB*, *PHABULOSA*; *SAC*, *suppressor of ACL5*; SAM, *S*-adenosyl methionine; SPDS, spermidine synthase; SPMS, spermine synthase; uORF, upstream open reading frame

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[14]. Isolation and characterization of *suppressor-of-acl5* (*sac*) mutants, which more or less suppress the dwarf phenotype of *acl5*, suggest that thermospermine may have a role in the upstream open reading frame (uORF)-mediated translational control of a subset of genes including *SAC51*, a gene encoding a potential negative regulator of vascular differentiation [15,16]. However, the precise mode of action of thermospermine remains to be understood. Because the *Arabidopsis spms* mutant, which is defective in spermine synthase, is wild type in appearance [17], the structural difference between thermospermine and spermine must be critical for their respective functions. We therefore addressed structural features of polyamines that are required for the control of plant vascular differentiation and stem elongation. Here we report our finding that norspermine, which has the C3C3 arrangement of carbon chains in common with thermospermine (Fig. 1), can functionally replace thermospermine.

2. Materials and methods

2.1. Chemicals

All polyamines used in this study were hydrochloride salts. Spermine, spermidine, and norspermidine were purchased from Sigma–Aldrich. Thermospermine and homocaldopentamine were provided by Masaru Niitsu, Josai University, Japan [18]. Norspermine was chemically synthesized according to the method described previously [19].

2.2. Plant materials and growth condition

The *A. thaliana* mutant strains *acl5-1* and *spms-1* were as previously described [11,17]. All mutants and the wild type used in this study were in the Landsberg *erecta* background. Plants were grown on rockwool cubes or Murashige-Skoog liquid or agar media supplemented with 3% sucrose at 22 °C under continuous fluorescent light. For daily treatment of plants with a polyamine, 40 μl of the respective 0.1 mM polyamine solution was added to the shoot apex of *acl5-1 spms-1* from 10 days after germination. The xylogenic culture of *Zinnia elegans* mesophyll cells was done as described [20].

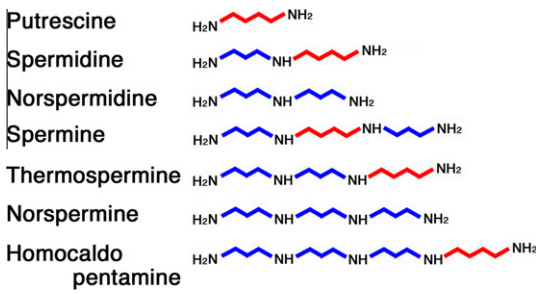

Fig. 1. Molecular structures of the three major polyamines and the polyamines used in this study. Red and blue lines indicate carbon chains of C3 and those of C4, respectively.

2.3. RNA extraction and expression analyses

Total RNA was extracted from whole seedlings by the SDS–phenol method [11] and cDNA was synthesized by using the PrimeScript reverse transcription reagent kit (Takara, Kyoto, Japan). Real-time PCR was performed by using the DNA Engine Opticon2 System with iQ SYBR Green Supermix (Bio-Rad). Gene-specific primers are shown in Supplementary Table S1. *ACTIN8* (At1g49240) was used to normalize the reaction. The specificity of the PCR was confirmed by melting curve analysis. GUS activity was detected by fluorometric and histochemical assays as described previously [15].

3. Results

3.1. Norspermine partially rescues the stem elongation defect of acl5

To compare the effect of norspermine with that of thermospermine and spermine, we used *acl5-1 spms-1* double mutants, which produce neither thermospermine nor spermine. Our experiments revealed that daily application of norspermine and thermospermine but not of spermine to shoot tips of *acl5-1 spms-1* seedlings clearly rescued the stem growth defect (Fig. 2). We confirmed that, under the same growth condition, application of these polyamines to wild type seedlings had no effect on the appearance of the adult flowering plants.

3.2. Norspermine and thermospermine down-regulate ACL5 and SAMDC4/BUD2

To know the effect of norspermine at the molecular level, transcript levels of the genes involved in the synthesis and catabolism of polyamines were investigated. Norspermine, thermospermine, or spermine was added to liquid cultures of *acl5-1 spms-1* seedlings. Quantitative reverse transcription-PCR experiments revealed that the level of the *acl5-1* transcript in *acl5-1 spms-1*, which is upregulated by a probable feedback response to thermospermine deficiency [12], was drastically reduced by norspermine as well as by thermospermine (Fig. 3A). The *ACL5* expression in the wild type was also reduced by norspermine and thermospermine but not by spermine. Because *spms-1* represents a T-DNA insertion allele in the 5′ leader sequence of *SPMS* and contains no detectable

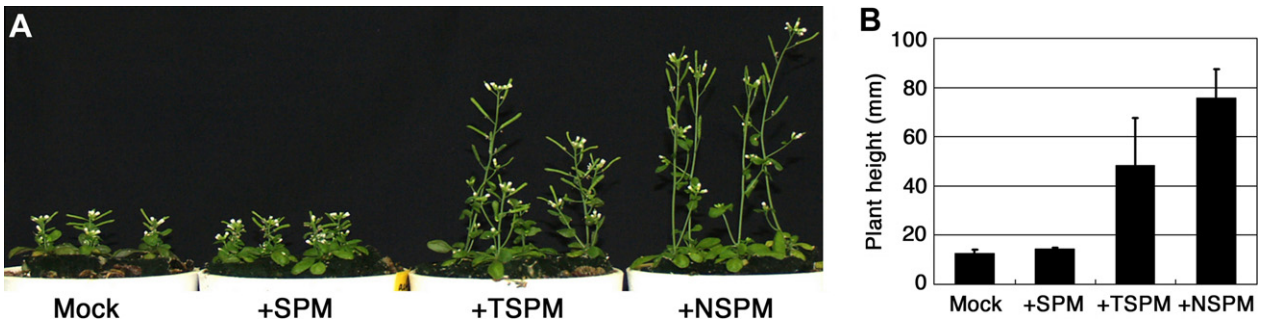

Fig. 2. Effects of exogenous application of polyamines on the growth of *acl5-1 spms-1* mutants. (A) Phenotypes of 40-day-old *acl5-1 spms-1* plants. Spermine (SPM), thermospermine (TSPM), or norspermine (NSPM) was applied to the shoot tip of the plants everyday from 10 days after germination as 40 μl of the 0.1 mM solution. (B) Plant height of 40-day-old *acl5-1 spms-1* plants treated as in (A). Error bars represent S.D. values of three independent experiments.

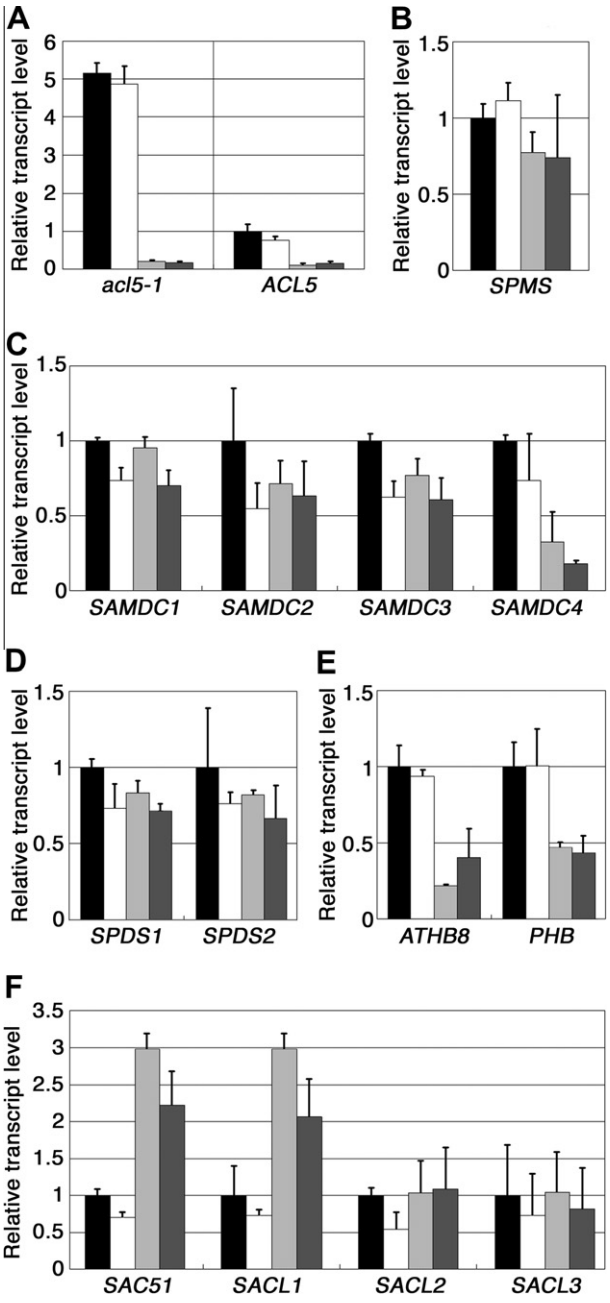

Fig. 3. Effects of exogenous application of polyamines on gene expression. Relative transcript levels of *ACL5* (A), *SPMS* (B), *SAMDC* genes (C), *SPDS* genes (D), HD-ZIP III genes (E), and *SAC51*-like genes (F) were examined by quantitative RT-PCR. Wild type (the right panel of A and B) and *acl5-1 spms-1* (the left panel of A, C, D, E, and F) seedlings were grown for 10 days in liquid MS medium and treated with mock (black bars), spermine (white bars), thermospermine (light gray bars), and norspermine (dark gray bars) at 0.1 mM for 24 h, respectively. Transcript levels were set to 1 in mock-treated controls. *acl5-1* in the left panel of (A) represents the mutant transcript derived from the *acl5-1* allele. Error bars represent S.D. values of three independent experiments.

transcripts for *SPMS*, we examined the transcript level of *SPMS* in wild type seedlings and confirmed that *SPMS* expression was neither responsive to norspermine, spermine, nor thermospermine treatment (Fig. 3B). The *Arabidopsis* genome contains four genes encoding SAM decarboxylase, *SAMDC1* to *SAMDC4/BUD2* [21]. We found that, among the four genes, only *SAMDC4/BUD2* showed much higher expression in *acl5-1 spms-1* mutants than in the wild type and this was attributed to the effect of the *acl5-1* allele (Supplementary Fig. S1). *SAMDC4/BUD2* was significantly down-regulated by norspermine and thermospermine (Fig. 3C). Transcript levels of *SPDS1* and *SPDS2*, two genes encoding SPDS, are not affected in *acl5-1 spms-1* [17]. These genes were not responsive to exogenous spermine, thermospermine, and norspermine in *acl5-1 spms-1* (Fig. 3D). We further examined transcript levels of all the five genes encoding PAO, *PAO1* to *PAO5*, and found that *acl5-1 spms-1* mutants had normal levels of all of these *PAO* transcripts (Supplementary Fig. S2A). They were also not altered by the three tetramines (Supplementary Fig. S2B).

3.3. Norspermine and thermospermine affect the expression of key genes for vascular development

We next examined expression of the genes involved in the regulation of vascular differentiation. ATHB8 and PHABULOSA (PHB) are members of the class III homeodomain-leucine zipper (HD-ZIP III) proteins which are known to be essential for vascular development [22,23]. Transcripts of these genes are expressed at much higher levels in *acl5-1* compared to the wild type [13,15]. Our experiments revealed that *ATHB8* and *PHB* were also down-regulated by both norspermine and thermospermine in *acl5-1 spms-1* (Fig. 3E).

Our previous study identified a bHLH-type transcription factor SAC51 as a probable negative regulator of vascular differentiation because the suppression of the *acl5-1* phenotype by a dominant suppressor allele, *sac51-d*, is attributed to overexpression of *SAC51* [15]. The *SAC51* transcript level is up-regulated by thermospermine [13]. The *Arabidopsis* genome contains three additional genes that are closely related to *SAC51*; At5g09460, At5g50010, and At1g29950. Hereafter we name these *SACL1, SACL2,* and *SACL3*, respectively. *acl5-1 spms-1* mutants showed normal levels of these transcripts (Supplementary Fig. S3). We found that transcript levels of *SAC51* and *SACL1* were up-regulated by both thermospermine

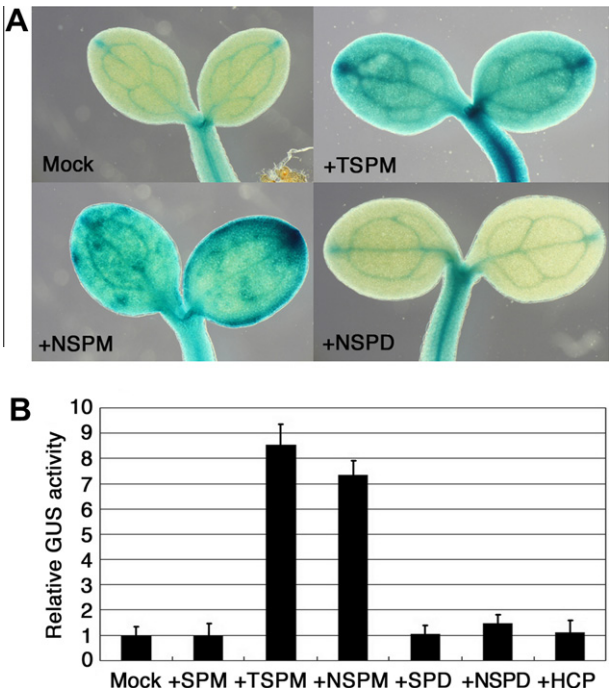

Fig. 4. Effects of exogenous application of polyamines on the *SAC51-GUS* fusion gene expression. *acl5-1 spms-1* seedlings carrying the *GUS* reporter gene fused with the *SAC51* promoter and its 5′ leader region (15) were grown for 3 days in MS solutions, treated with each polyamine at 0.1 mM for 24 h, and stained (A) or assayed (B) for GUS activity. SPM, spermine; TSPM, thermospermine; NSPM, norspermine; SPD, spermidine; NSPD, norspermidine; HCP, homocaldopentamine.

and norspermine while those of *SACL2* and *SACL3* were not altered by these polyamines (Fig. 3F).

3.4. Norspermidine cannot substitute for thermospermine in regulating stem elongation

The C3C3 arrangement of carbon chains present in thermospermine and norspermine is also contained in a triamine, norspermidine (Fig. 1). Unlike thermospermine and norspermine, however, norspermidine did not rescue the dwarf phenotype of *acl5-1* by its daily application to the shoot apex. We also examined expression of *SAC51* in *acl5-1 spms-1* mutants by using the GUS reporter gene fused to the promoter and the 5′ leader sequence of *SAC51* [15]. GUS staining is detected in most tissues but preferentially in vascular tissues in wild type seedlings [15], and only weak staining is detected in *acl5-1 spms-1* seedlings (Fig. 4A). External norspermidine, spermidine, and spermine at 0.1 mM had no inductive effect on the GUS activity, while thermospermine and norspermine at 0.1 mM increased it drastically in *acl5-1 spms-1* seedlings (Fig. 4B). Because the efficacy of triamines to influence biological events may be generally lower than that of tetramines, we examined the effect of norspermidine at higher concentrations (0.2–2 mM) but observed no influence on the GUS activity (Supplementary Fig. S4). We further examined the effect of homocaldopentamine (C3C3C3C4, Fig. 1) on the GUS activity but no effect was detected at 0.1 mM (Fig. 4B).

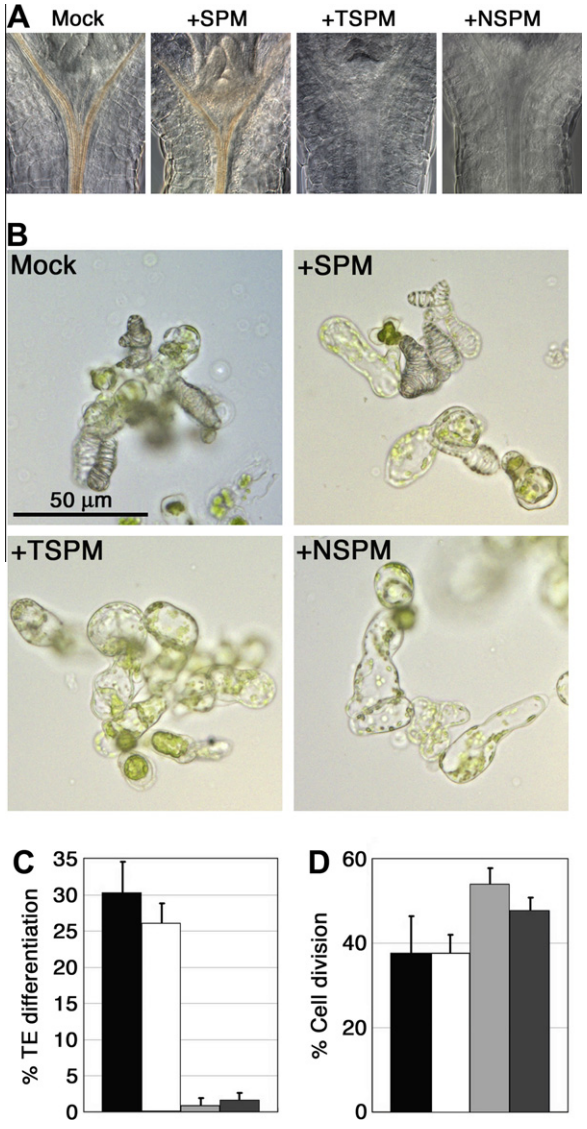

Fig. 5. Inhibition of xylem differentiation by thermospermine and norspermine. (A) Effects of polyamines in the growth medium on xylem vessel differentiation in *acl5-1 spms-1*. Seedlings were grown for 7 days in the liquid MS medium supplied with each polyamine at 10 μM. Hypocotyls were observed under light microscopy. (B–D) Effects of polyamines in the growth medium on tracheary element differentiation in *Zinnia* cultured cells. *Zinnia* mesophyll cells were cultured in the absence of polyamines (mock) or in the presence of 3 μM spermine, 3 μM thermospermine, or 0.1 μM norspermine for 4 days. Tracheary element differentiation (B and C) and cell division (D) were scored under light microscopy. Error bars in (C) and (D) represent S.D. values of three independent experiments.

3.5. Xylem differentiation is blocked by norspermine and thermospermine

Microscopic observation of the *acl5-1 spms-1* seedlings grown in the liquid MS medium supplemented with a polyamine showed that thermospermine and norspermine but not spermine resulted in an apparent reduction in the development of lignified vessel elements, which is observed as brown-colored tissues in Fig. 5A, suggesting inhibitory effects of these polyamines on xylem vessel differentiation. Then, we examined the effect of these polyamines on an in vitro *Z. elegans* xylogenic culture system, where single mesophyll cells transdifferentiate into tracheary elements [24]. We found that addition of 3 μM thermospermine or 0.1 μM norspermine to *Zinnia* cell cultures strongly blocked the transdifferentiation but addition of 3 μM spermine had no such effect (Fig. 5B and C). These concentrations of these polyamines did not affect the rate of cell division in this culture system (Fig. 5D).

4. Discussion

Norspermidine and norspermine have been detected in a few plant species including alfalfa and cotton, and are predicted to be synthesized successively by each specific APT or a single APT with broad substrate specificity from 1,3-diaminopropane, which is produced by degradation of spermidine or spermine by PAO [25]. However, because there are no putative genes for such APT identified in the *Arabidopsis* whole genome sequence, it is unlikely that *Arabidopsis* tissues contain these uncommon polyamines. With the use of *Arabidopsis* mutants deficient in the synthesis of spermine and thermospermine, we demonstrated that norspermine can function as a substitute for thermospermine in the promotion of stem elongation, the repression of lignified vessel differentiation, and the regulation of a subset of genes. The *Arabidopsis spms* mutant has been shown to be more sensitive to high salt and drought conditions than the wild type [26] but our preliminary experiments revealed that the *acl5-1 spms-1* mutant seedlings grown in the presence of norspermine showed no obvious increase in the tolerance to these stresses, suggesting that norspermine may not substitute for spermine. On the other hand, norspermidine and homocaldopentamine did not substitute for thermospermine in our experiments. These results suggest that, in addition to the C3C3 structure of carbon chains, four amino moieties are important for the action of thermospermine and norspermine. It is possible, however, that the different effects of each exogenous polyamine are due to different uptake efficiencies. Polyamine transport systems remain to be elucidated in plants. Thermospermine and norspermine were also shown to almost completely block transdifferentiation of mesophyll cells to tracheary elements in the *Zinnia* culture. Interestingly, norspermine showed an inhibitory effect on the transdifferentiation of *Zinnia* cells at lower concentrations than did thermospermine. It will be also of interest to determine whether other uncommon tetramines and branched ones like tris(3-aminopropyl)amine could substitute for thermospermine or not. Such studies should give insight into an optimal structure of polyamines required for *ACL5*-dependent growth.

This study revealed that expression of one of the four *SAMDC* genes, *SAMDC4/BUD2*, was increased in *acl5-1* mutants and down-regulated by thermospermine and norspermine. This is reminiscent of the *ACL5* expression, which is under negative feedback control by thermospermine [13]. Because the *bud2* mutant shows bushy and dwarf phenotype [21], it is possible that the supply of dcSAM for the synthesis of thermospermine is mediated predominantly by *SAMDC4/BUD2* and that the *bud2* mutant lacks thermospermine, resulting in the dwarfism. *SAMDC1* has two uORFs and its translation is down-regulated by excess spermidine and spermine through the uORF-mediated pathway [27]. *SAMDC2* and *SAMDC3* also have the two conserved uORFs but *SAMDC4/BUD2* does not [28]. Detailed studies of the *bud2* mutant and *BUD2* expression profiles are needed to clarify the relation between *ACL5* and *SAMDC4/BUD2*.

The "uncommon" polyamines such as thermospermine, norspermine, and other longer or branched polyamines, were initially detected in thermophilic bacteria and have been implicated in nucleic acid stabilization and mRNA translation under extreme growth conditions [29,30]. Our previous studies suggest that thermospermine plays a role in overcoming the inhibitory effect of the uORFs of the *SAC51* transcript on the main ORF translation, although the precise mode of action is as yet to be elucidated [15,16]. If so, it is likely that the observed increase in the *SAC51* transcript level by thermospermine and norspermine represents translation-dependent stabilization of existing transcripts rather than new transcription. A peptide sequence similar to that encoded by the longest uORF of *SAC51* is conserved within that of *SACL1*, *SACL2*, and *SACL3*, but *SACL2* and *SACL3* showed no clear increase in their transcript levels in response to thermospermine and norspermine. Thus, independent of the conserved peptide sequences encoded by these uORFs, thermospermine and norspermine might act on specific RNA sequences and enhance the main ORF translation. Because *ACL5* is highly expressed in provascular cells [14], thermospermine may be responsible for specifying tissues that express a subset of genes including *SAC51* and *SACL1*. Further identification of the target genes whose transcript level is affected by thermospermine and norspermine will help to elucidate the action mechanisms of these tetramines. Finally, we should also note the possibility that the phenomena described above are due to versatile actions of exogenous polyamines and specific modification or oxidation of thermospermine and norspermine could play a role in the observed effects.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.05.035.

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