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Spermine is not essential for survival of *Arabidopsis*

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Abstract Spermine is the final product of the polyamine biosynthetic pathway and is ubiquitously present in most organisms. The genome of *Arabidopsis thaliana* has two genes encoding spermine synthase: *ACAULIS5* (*ACL5*), whose loss-of-function mutants show a severe defect in stem elongation, and *SPMS*. In order to elucidate the function of spermine in plants, we isolated a T-DNA insertion mutant of the *SPMS* gene. Free and conjugated spermine levels in the mutant, designated *spms-1*, were significantly decreased compared with those in the wild-type, but no obvious morphological phenotype was observed in *spms-1* plants. We further confirmed that *acl5-1 spms-1* double mutants contained no spermine. Surprisingly, *acl5-1 spms-1* was fully as viable as the wild-type and showed no phenotype except for the reduced stem growth due to *acl5-1*. These results indicate that spermine is not essential for survival of *Arabidopsis*, at least under normal growth conditions.

Key words: ACL5; Polyamine; Spermine synthase; SPMS; *Arabidopsis thaliana*

1. Introduction

Polyamines (putrescine, spermidine and spermine) are ubiquitous low-molecular-mass polycations involved in many cellular processes, including chromatin condensation, maintenance of DNA structure, RNA processing, modulation of enzyme activities, stabilization of membranes, and scavenging of free radicals [1–3]. In higher plants, polyamines are known to act as modulators of shoot morphogenesis, flowering, longevity, fruit ripening, and stress responses [4–9]. Since high intracellular polyamine levels are detrimental to cells [10], the levels are finely regulated by biosynthesis, degradation, and transport systems. In animal and fungal cells, ornithine decarboxylase (ODC) is the first and rate-limiting enzyme in the synthesis of endogenous polyamines. ODC catalyzes the conversion of ornithine into putrescine, which is the precursor for higher polyamines. Plant and some bacterial cells have an additional indirect route to putrescine from arginine. Recently, all of the genes involved in polyamine biosynthesis in *Arabidopsis thaliana* have been identified (Fig. 1) [11–13]. Putrescine synthesis in *Arabidopsis* is uniquely dependent on the arginine route to putrescine due to loss of its *ODC* gene [14]. Spermidine and spermine are synthesized by successive transfer of the aminopropyl moiety of decarboxylated *S*-adenosylmethionine to the secondary amines of putrescine.

A number of studies have indicated that polyamines are essential for normal cell growth and differentiation. Furthermore, isolation and characterization of mutants defective in polyamine biosynthesis from several organisms have revealed the significance of each polyamine molecule. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, null mutants of a gene encoding *S*-adenosylmethionine decarboxylase have an absolute requirement for spermidine or spermine for growth and cell cycle progression [15,16]. The *SPE4* gene encoding spermine synthase in *S. cerevisiae* is not essential for normal growth [17]. Similarly, in mouse fibroblast cell cultures, disruption of a spermine synthase gene that results in a lack of spermine and a greater accumulation of spermidine was shown to have no significant effect on the growth of cells [18]. In contrast, a splice mutation of a spermine synthase gene in humans has recently been shown to be associated with Snyder–Robinson syndrome, an X-linked mental retardation disorder [19]. Affected males have low levels of intracellular spermine in lymphocytes and fibroblasts, and the observed clinical features may be a result of cerebellar dysfunction and a defective functioning of red nucleus neurons, which normally contain high levels of spermine [19]. In bacterial cells, the predominant polyamines are putrescine and spermidine. The *Escherichia coli* genome has no gene for spermine synthase. Furthermore, *E. coli* mutants that cannot synthesize spermidine because of deletions in the gene encoding *S*-adenosylmethionine decarboxylase are still able to grow at near normal rates in purified media deficient in polyamines [20]. These findings suggest that each polyamine molecule has a specialized function in multicellular systems.

In higher plants, however, there have been few studies on mutations in genes that are involved in polyamine metabolism. Ethyl methanesulfonate-induced mutants of *Arabidopsis*, which have reduced ADC activities, have been shown to exhibit excess lateral root branching [21], but the gene for the mutant phenotype remains to be identified. On the other hand, analysis of an *En-1* transposon mutant of *ADC2* has revealed differential inducibility of the two *Arabidopsis ADC* genes by osmotic stress [22]. The *Arabidopsis* genome has two genes encoding spermidine synthase, *SPDS1* and *SPDS2*, and two genes encoding spermine synthase, *ACAULIS5* (*ACL5*) and *SPMS* [23–25]. In a previous study, loss-of-function mutations of the *ACL5* gene have been shown to result in a

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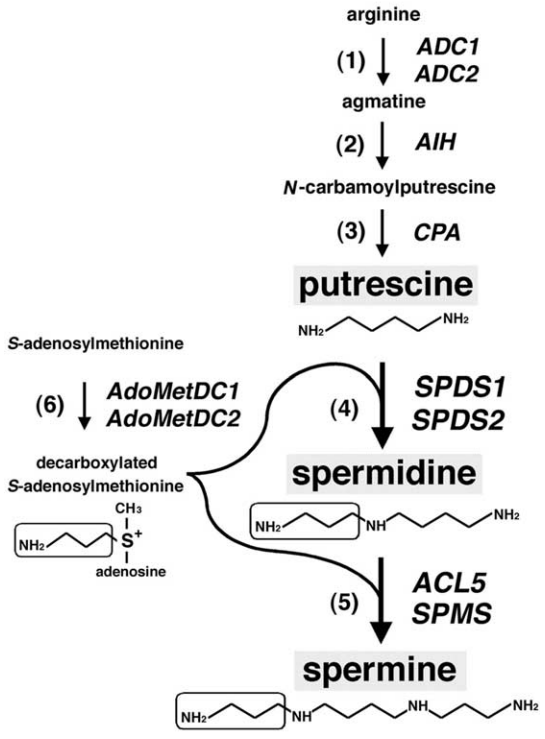

Fig. 1. Pathway of biosynthesis of the major polyamines (putrescine, spermidine and spermine) in *Arabidopsis*. Enzymes shown in numbers are (1) arginine decarboxylase (ADC; EC 4.1.1.19), (2) agmatine iminohydrolase (AIH; EC 3.5.3.12), (3) *N*-carbamoylputrescine amidohydrolase (CPA; EC 3.5.1.53), (4) spermidine synthase (SPDS; EC 2.5.1.16), (5) spermine synthase (SPMS; EC 2.5.1.22), and (6) *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50). All of the related genes identified in the *Arabidopsis* genome are shown in italic type.

severely dwarfed phenotype [23]. To elucidate further the significance of spermine in *Arabidopsis*, we isolated a T-DNA insertion mutant of the *SPMS* gene and generated *acl5 spms* double mutants. We show here that spermine is not essential for survival of *Arabidopsis*.

2. Materials and methods

2.1. Plant material and growth conditions

The Columbia (Col-0) ecotype of *A. thaliana* (L.) Heynh was used as the wild-type. The *acl5-1* allele in the Landsberg *erecta* background [26] was backcrossed seven times into the Columbia ecotype. Plants were grown under continuous illumination at 22°C on rock-wool bricks supplemented with vermiculite or on 0.8% (w/v) agar plates containing Murashige–Skoog (MS) salts (pH 5.8) and 3% sucrose after surface sterilization of seeds.

2.2. Identification of the spms-1 T-DNA insertion mutant

DNA pools of the *Arabidopsis* (ecotype Col-0) T-DNA insertion lines deposited at the Kazusa DNA Research Institute were screened for the presence of a mutant in the *SPMS* locus. The T-DNA left border primer, LB (5′-ATAAC GCTGC GGACA CATCT AC-3′), was used for screening in combination with each of two primers, SPMSF (5′-GAACC GCACA ATGAA ATACA GTT-3′) and SPMSR (5′-AGACA TACTT TCAGG TACTA CTC-3′), which were designed according to the 5′ and 3′ sequences of the *SPMS* gene. A T-DNA insertion site in the *spms-1* allele was determined by sequencing the resulting positive polymerase chain reaction (PCR) fragment. Plant genotyping for the *spms-1* allele was performed by PCR using the LB primer and the SPMSF gene-specific primer described above.

2.3. Gene expression analysis

Total RNA was extracted from 16-day-old seedlings grown on MS agar plates according to the sodium dodecyl sulfate (SDS)–phenol method [27]. Reverse transcriptase-mediated PCR (RT-PCR) was conducted by using an RNA LA PCR Kit (Takara, Kyoto, Japan) with 0.5 μg of total RNA. An oligo(dT) adapter primer (5′-GCGGCC-GCT$(dT)_{18}$-3′) was used for first-strand cDNA synthesis. Primers for the *SPMS* transcript were SPMSF′ (5′-TGTGC ATATC AG-GAG ATGAT AGC-3′) and SPMSR′ (5′-TCCTC TTCAA GAGTT CTACA AAG-3′). PCR conditions were 40 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 2 min. As a control, the *Actin8* transcript was amplified for 25 PCR cycles using a pair of primers, ACT8F (5′-TGAGC CAGAT CTTCA TCGTC-3′) and ACT8R (5′-TCTCT TGCTC GTAGT CGACA-3′) [28].

For RNA gel blot analysis, 10 μg of each total RNA sample was separated on 1.2% agarose–formaldehyde gels and blotted onto Hybond-N^+ membranes (Amersham). Gene-specific probes for *AdoMetDC1* and *AdoMetDC2* were prepared by PCR using *Arabidopsis* genomic DNA as a template with primer pairs Ado1F (5′-AGATG GCCTT ATCTG CAATC GG-3′) and Ado1R (5′-CTAGA TTCCC TCGTC CTTCT CGT-3′) for *AdoMetDC1* and Ado2F (5′-GCAGT GGGAT ATGAT TTCAC-3′) and Ado2R (5′-TGACC TTGTT AACTA TGAGG-3′) for *AdoMetDC2*. The probes of *ACL5*, *SPMS*/*SPDS3*, *SPDS1*, and *SPDS2* were prepared as described previously [24]. These probes were ^{32}P-labeled by random-primed synthesis (Takara). The blots were hybridized at 42°C for 16 h with a labeled probe in 50% (v/v) formamide, 10% (w/v) dextran sulfate, 1 M NaCl and 1% SDS and then washed twice with 2×sodium saline citrate (SSC), 0.1% SDS at 65°C for 30 min and once with 0.1×SSC at room temperature for 5 min. The membranes were exposed to X-ray films at −80°C for 72 h.

2.4. Polyamine analysis

In order to extract polyamines, 16-day-old seedlings (0.2 g fresh weight) were homogenized in 2 ml of 5% (w/v) perchloric acid (PCA) containing 1 nmol of 1,6-hexanediamine as an internal standard. After centrifugation, the supernatant was preserved and the pellet was resuspended in 5% PCA after several washes with the same solution. Aliquots of acid-soluble and acid-insoluble fractions, containing free plus conjugated polyamines and bound polyamines, respectively, were subjected to hydrolysis in 6 M HCl at 110°C for 18 h to convert the conjugated and bound forms to a free form. After the hydrolysate was dried in vacuo at 70°C, the residues were dissolved in 500 μl of 5% PCA. Aliquots (each 100 μl) were added to 200 μl of saturated sodium carbonate and 200 μl of dansyl chloride (5 mg/ml acetone). After brief vortexing, the mixture was incubated in darkness at 30°C for 16 h. Excess dansyl reagent was inactivated by the addition of 50 μl of 0.9 M proline. Dansylated polyamines were extracted in 0.5 ml toluene, dried in a Speed-Vac concentrator (Savant), redissolved in 50 μl of toluene, and analyzed by high performance liquid chromatography (HPLC) using a Wakosil-II 5C18 HG reverse-phase column (particle size, 5 μm; 4.6×150 mm; Wako, Osaka, Japan). Portions (20 μl) of the polyamine fractions were applied to the column and eluted with a programmed methanol:water solvent gradient, changing from 55% to 85% over a period of 15 min at a flow rate of 0.8 ml/min. Elution was completed after 15 min. Polyamines were quantified by a fluorescence detector set at excitation and emission wavelengths of 365 and 510 nm, respectively. Conjugated polyamine content was calculated by subtracting free polyamine content from total acid-soluble polyamine content. Results were standardized with equimolar (0.5 nmol) mixtures of dansylated polyamines.

3. Results

3.1. Isolation of the spms-1 T-DNA insertion mutant

Previous studies have revealed that the *Arabidopsis* genome contains only four members of the conserved aminopropyl transferase gene family, *SPDS1*, *SPDS2*, *ACL5* and *SPMS*, and that their gene products are enzymatically active in yeast and in vitro [23–25]. *SPMS*, which was tentatively named *SPDS3* in our previous paper [24], may be a paralog for the spermine synthase gene because, in phylogenetic analysis, SPMS shares a branch with the known plant spermidine syn-

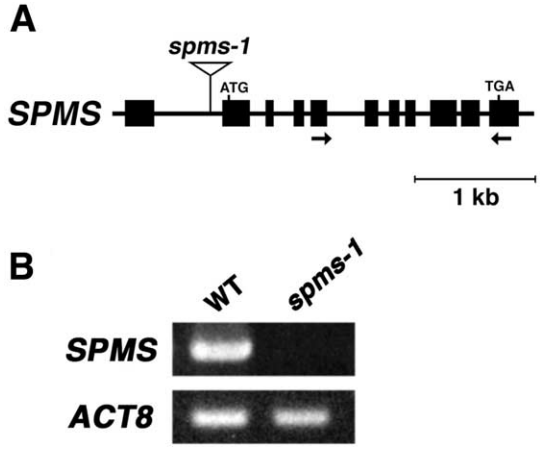

Fig. 2. Molecular characterization of the *spms-1* T-DNA insertional mutant. A: Schematic diagram of the *spms-1* allele indicating the location of the T-DNA insertion. The positions and lengths of exons and introns are indicated by closed rectangles and lines, respectively. Arrows indicate the *SPMS*-specific primers used in RT-PCR analysis shown in B. B: RT-PCR analysis of *SPMS* expression in wild-type (WT) and *spms-1* plants. Total RNA was prepared from 16-day-old seedlings. The level of *Actin8* (*ACT8*) was used as an internal control.

thases that is divergent from the spermine synthase cluster including ACL5 [25].

To genetically dissect the in vivo function of SPMS, we identified a mutant of the *SPMS* gene by screening a population of T-DNA insertion lines from the Kazusa DNA Research Institute. The allele, designated *spms-1*, contains a T-DNA insertion in the first intron, 180 bp upstream of the translational start codon (Fig. 2A). RT-PCR analysis using the primers that were designed to amplify a part of the coding region downstream of the insertion site confirmed the absence of the *SPMS* full-length transcripts in homozygous *spms-1* plants (Fig. 2B), suggesting that *spms-1* represents a null allele. We detected no obvious phenotypic changes in homozygous *spms-1* plants compared with wild-type plants under normal growth conditions (Fig. 3).

3.2. *Characterization of the* acl5-1 spms-1 *double mutant*

To determine whether no phenotype of *spms-1* could be attributed to genetic redundancy between ACL5 and SPMS, we generated *acl5-1 spms-1* double mutant plants by crossing *acl5-1* and *spms-1*. The *acl5-1* mutant has a substitution of a well-conserved amino acid within the catalytic domain of spermine synthase and has been suggested to be a loss-of-function allele [23]. Surprisingly, the *acl5-1 spms-1* double

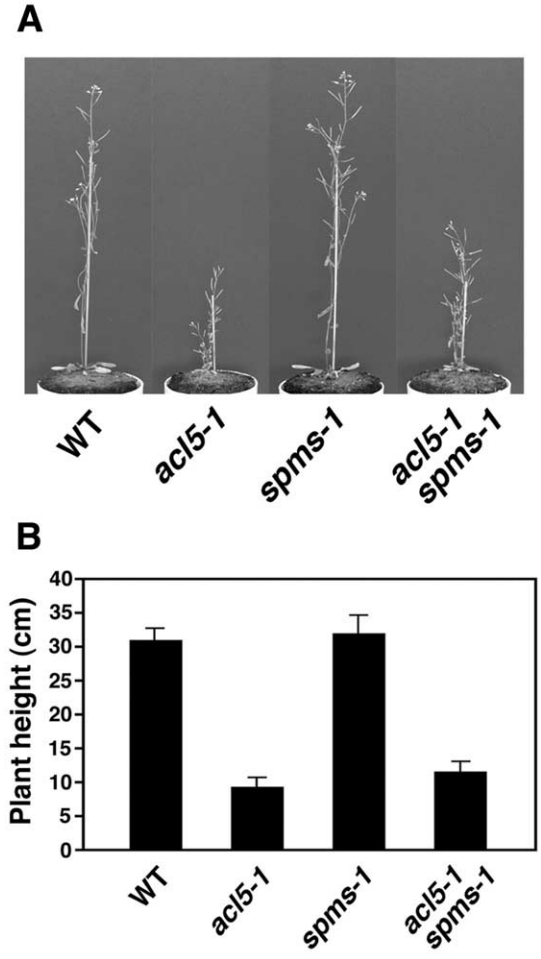

Fig. 3. Phenotype of the *acl5-1*, *spms-1*, and *acl5-1 spms-1* mutants. A: Comparison of the gross morphology of wild-type (WT), *acl5-1*, *spms-1*, and *acl5-1 spms-1* plants. B: Plant heights of 6-week-old plants. Bars indicate ±S.E.M. ($n = 10$).

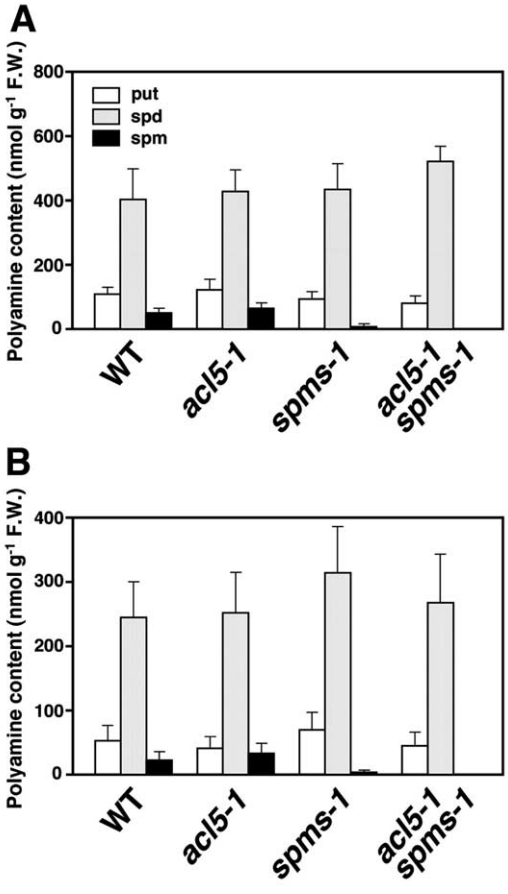

Fig. 4. Polyamine contents in wild-type (WT), *acl5-1*, *spms-1*, and *acl5-1 spms-1* seedlings. A: Free polyamines. B: Polyamines conjugated in the PCA-soluble fraction. Polyamines were extracted from 16-day-old seedlings of each plant and quantified by HPLC. The limit of detection for spermine was 5 nmol/g fresh weight seedlings. Bars indicate ±S.E.M. ($n = 3$). put, putrescine; spd, spermidine; spm, spermine.

mutants were short in stature but indistinguishable from *acl5-1* single mutant plants (Fig. 3).

We further measured endogenous levels of free and conjugated polyamines in *acl5-1*, *spms-1*, and *acl5-1 spms-1* double mutant seedlings. As shown in Fig. 4A, no significant difference was detected between wild-type and *acl5-1* seedlings with respect to the levels of free forms of putrescine, spermidine, and spermine. The levels of conjugated forms of these polyamines were also unaltered in *acl5-1* (Fig. 4B). On the other hand, free and conjugated spermine levels in *spms-1* were decreased to 5.8% and 3.4% of those in the wild-type, respectively. Furthermore, no spermine in the free and conjugated forms was detected in *acl5-1 spms-1* double mutants. These results indicate that only ACL5 and SPMS function as spermine synthase in *Arabidopsis*.

3.3. *Effects of* acl5-1 *and* spms-1 *on the expression of polyamine biosynthetic genes*

We examined the effects of *acl5-1* and *spms-1* mutations on the expression of polyamine biosynthetic genes by RNA gel blot analyses. A previous study has shown that *ACL5* expression is upregulated in *acl5-1* mutants [23]. Our results showed that the *ACL5* transcript level was increased in *acl5-1 spms-1* double mutants as well as in *acl5-1*, while it was unaffected in *spms-1* (Fig. 5). *SPMS* transcripts were probed with a fragment corresponding to the first exon of *SPMS*, which is upstream of the T-DNA insertion site of *spms-1*. Since possible truncated *SPMS* transcripts derived from a region upstream of the insertion site were not detected in *spms-1* and *acl5-1 spms-1*, they may be immediately degraded as aberrant transcripts. *acl5-1* appeared to have no effect on *SPMS* expression. Transcript levels of *SPDS1* and *SPDS2* were not affected by *acl5-1* and *spms-1* mutations. Transcript levels of *AdoMetDC1* and *AdoMetDC2*, which encode *S*-adenosylmethionine decarboxylase (Fig. 1) [29], were also unchanged in each mutant.

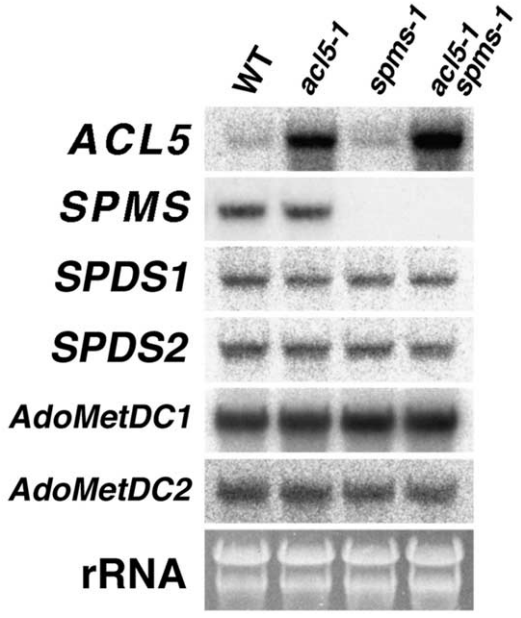

Fig. 5. RNA gel blot analysis of the expression of polyamine biosynthetic genes in *acl5-1*, *spms-1*, and *acl5-1 spms-1* mutants. Total RNA was prepared from 16-day-old wild-type (WT), *acl5-1*, *spms-1*, and *acl5-1 spms-1* seedlings. Each lane contained 10 μg of total RNA. rRNA is shown as a loading control.

4. Discussion

Our results revealed that free and conjugated spermine levels in *spms-1* were markedly reduced, while they were almost unchanged in *acl5-1*, indicating that SPMS plays a major role in spermine biosynthesis in *Arabidopsis*. Panicot et al. [25] confirmed the existence of coimmunoprecipitating SPDS1–SPDS2 and SPDS2–SPMS heterodimers in vivo. They also found by a direct two-hybrid test that SPMS could interact with SPDS1 and SPDS2, while no interaction of ACL5 with either SPDS1 or SPDS2 was found [25]. Taking into account the fact that spermidine is a substrate for spermine synthesis, a tight connection between SPDS and SPMS may favor SPMS as a major spermine synthase. It is surprising, however, that *spms-1* mutants show no aberrant phenotype in contrast to severely dwarfed *acl5-1* mutants. How is ACL5 solely involved in stem elongation? One possibility is that the ACL5 protein specifically interacts with unidentified molecules. Such interactions might result in cellular localization of the synthesized spermine or formation of certain spermine conjugates required for stem elongation. Plant polyamines are most commonly conjugated to cinnamic acids such as *p*-coumaric, ferulic and caffeic acids. The resulting conjugates are known as hydroxycinnamic acid amides and have been implicated in detoxicating phenolic compounds known to inhibit growth [30]. Spermine availability in certain conjugated forms might be filled only through the ACL5 function. Alternatively, it is possible that ACL5 has additional enzyme activity. However, we detected no obvious difference in the HPLC profile of polyamines between wild-type and *acl5-1* cell extracts (data not shown).

We found that *acl5-1 spms-1* double mutants have no detectable level of spermine but show no phenotype except for reduced stem growth caused by *acl5-1* mutation. Thus, we conclude that spermine is not essentially required for survival of *Arabidopsis*. Considering the large body of evidence for roles of polyamines in a wide range of biological processes in plants, most of the roles of spermine may be compensated by spermidine and/or putrescine. However, there have been some studies suggesting a specific role of spermine. In an in vitro experiment using nuclei of rice seedlings, a set of DNA binding proteins has been shown to be released from chromatin by low levels of spermine [31]. In the hypersensitive response (HR) of tobacco to tobacco mosaic virus (TMV) infection, there is a drastic increase in spermine levels in the intercellular spaces of the necrotic lesion-forming leaves [32]. Spermine was also shown to be a salicylate-independent endogenous inducer for acidic pathogenesis-related proteins conferring resistance to TMV infection [32]. Hiraga et al. found that a tobacco HR-induced peroxidase gene is responsive to spermine [33]. Unlike *SPDS1* and *SPDS2*, the *SPMS* gene is upregulated by abscisic acid, while the *ACL5* gene is responsive to auxin [23,24]. These findings suggest that spermine may exert its specific effect under certain stress conditions. Effects of spermine deficiency in *acl5-1 spms-1* on growth under stress conditions and on responses to various external stimuli should be further investigated for a better understanding of the physiological role of spermine.

In contrast to *acl5-1* mutants showing an increased level of

the mutated *acl5* transcript, *spms-1* mutants were shown to have a normal level of the *ACL5* transcript. Thus, the negative feedback loop of *ACL5* expression proposed in a previous study [23] appears to be mediated by an ACL5-dependent reaction product, not by spermine in general. It remains to be determined whether *SPMS* expression is also under the control of a negative feedback loop. Our results showed that the spermine deficiency in *acl5-1 spms-1* had no effect on the expression of *SPDS1*, *SPDS2*, *AdoMetDC1*, and *AdoMetDC2*. We also confirmed that the transcript levels of the other polyamine biosynthetic genes shown in Fig. 1 were not influenced in *acl5-1 spms-1* (data not shown). However, the possibility cannot be ruled out that these genes are post-transcriptionally regulated by depletion of spermine. The *Arabidopsis AdoMetDC1* gene has been shown to be subject to its small upstream open reading frame-mediated translational control by polyamines [34]. The basis of polyamine homeostasis, which involves transport and oxidation of polyamines as well as post-transcriptional regulation, remains largely uncharacterized and will be a focus of future studies.

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