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Characterization of the spermidine synthase-related gene family in *Arabidopsis thaliana*

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Abstract The *Arabidopsis* genome contains four genes that encode proteins similar to both spermidine synthase and spermine synthase of other organisms. Our previous study revealed that one of these genes, designated *ACAULIS5* (*ACL5*), encodes spermine synthase and that its null mutation results in a severe defect in the elongation of stem internodes. Here we report the characterization of the other three genes, designated *SPDS1*, *SPDS2* and *SPDS3*. Our results showed that SPDS1 and SPDS2 possess spermidine synthase activity in yeast spermidine synthase-deficient mutants, but the enzyme activity of SPDS3 remained to be determined. RNA gel blot analysis revealed that all of these genes are expressed in all plant organs but show different responses to exogenous plant hormones, suggesting that they are involved in different aspects of growth by modulating the contents of polyamines in plant cells.

Key words: Gene expression; Polyamine; Spermidine synthase; *Arabidopsis thaliana*

1. Introduction

A group of aliphatic amines known as polyamines play essential roles in cell growth and proliferation. Many studies carried out using prokaryotes and eukaryotes have shown that these cationic substances interact with anionic macromolecules such as nucleic acids, phospholipids and certain proteins [1,2]. Because of this property, it has been suggested that polyamines stimulate growth and development through enhanced transcription and translation and through membrane stabilization. In higher plants, the polyamine content changes with development of the plant organs and in response to environmental stimuli such as potassium deficiency, salinity and osmotic stress [3–5]. Polyamines and the plant hormone ethylene share a common metabolic precursor, *S*-adenosyl-methionine (SAM). Because ethylene promotes senescence, polyamines have been suggested to act as antisenescence factors [6]. However, the underlying mechanisms of their actions remain a matter of speculation.

Most organisms produce the diamine putrescine directly from ornithine through the activity of ornithine decarboxylase (ODC). Plants and some bacteria have an alternative pathway that starts with arginine to give agmatine via arginine decarboxylase (ADC) and then converts agmatine into putrescine through the successive activities of agmatine iminohydrolase and *N*-carbamoylputrescine amidohydrolase [4]. Genes involved in the biosynthetic pathways for polyamines have been cloned from several plant species and shown to be developmentally regulated [7]. The *Datura ODC* gene and the pea *ADC* gene have been found to be expressed at high levels in rapidly growing tissues, consistent with a role of polyamines in cell proliferation [8,9]. Interestingly, *Arabidopsis thaliana* has no *ODC* gene sequences and therefore lacks ODC activity [10]. Putrescine is metabolized to spermidine and spermine through the successive activities of spermidine synthase and spermine synthase with the use of decarboxylated SAM as an aminopropyl donor. In a previous study, we found that loss-of-function mutations of the *Arabidopsis ACAULIS5* (*ACL5*) gene, which encodes a spermine synthase, result in a severely dwarfed phenotype because of a specific defect in the cell growth in stem internodes [11]. The finding that *ACL5* expression was upregulated in *acl5* mutant plants suggests the presence of a negative feedback mechanism of spermine synthesis in plant cells.

The goal of our investigations is complete elucidation of the genetic control of polyamine synthesis in higher plant species. In this paper, we report the results of cDNA cloning and initial molecular analysis of all of the spermidine synthase-related genes in *Arabidopsis*.

2. Materials and methods

2.1. Plant growth conditions

The wild-type *A. thaliana* used in this study is ecotype Columbia unless otherwise stated. The *acl5-1* mutant allele is ecotype Landsberg *erecta* and has been described previously [11,12]. Plants were grown under continuous illumination at 22°C on vermiculite or on agar plates containing Murashige–Skoog (MS) salts and 3% sucrose after surface sterilization of seeds. For plant hormone treatment, seedlings were grown for 10 days in an aerated solution of MS salts with 3% sucrose.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR), cloning and sequencing

Total RNA was prepared using a SDS–phenol extraction method

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[13]. The first cDNA strand was synthesized using an RT-PCR kit (Takara, Kyoto, Japan) with an oligo-dT primer. RT-PCR primers were designed to amplify cDNA fragments containing complete coding regions of each gene. They were S1F (5′-CCATG GACGC TAAA G AAAC-3′) and S1R (5′-CAACG ATCCT CCAGA TTAG-3′) for *SPDS1*, S2F (5′-CCATG TCTTC AACAC AAGAA G-3′) and S2R (5′-CACTT CCACA TGAAG TTTCT C-3′) for *SPDS2*, and S3F (5′-CTATG GAGGG AGACG TC-3′) and S3R (5′-GATAT GGTAG AGCCA AACAG-3′) for *SPDS3*. PCR was performed with annealing at 55°C for 35 cycles, and the amplification products were cloned into the plasmid vector pGEM-T Easy (Promega). DNA sequences were determined by the use of a dye terminator cycle sequencing kit (Perkin Elmer).

2.3. RNA gel blot analysis

RNA samples were electrophoresed in formaldehyde gels. Transfer to GeneScreen Plus (NEN) nylon membranes, hybridization and washes were carried out according to the manufacturer's instructions. Gene-specific probes for *SPDS1* and *SPDS2* were prepared by PCR using *Arabidopsis* genomic DNA as a template with primer pairs, S1F′ (5′-TTCGC CAAGA AGGTC ATTGA GT-3′) and S1R′ (5′-TGT-TA ATGTT CTTGG TCTTC GG-3′) for *SPDS1* 3′-UTR, and S2F′ (5′-TGCTA AGAAG GTGAT TGATT CG-3′) and S2R′ (5′-TAACG TCAAC GTCGA CAATA CC-3′) for *SPDS2* 3′-UTR. The *SPDS3* probe was a cDNA fragment cloned as described above. The *ACL5* probe has been described previously [11]. These fragments were ^{32}P-labeled by random-primed synthesis (Takara).

2.4. Expression in yeast and bacterial cells

The cDNA fragments of *SPDS1*, *SPDS2*, *SPDS3* and *ACL5* cloned into the pGEM-T Easy vector were transferred into the *Eco*RI restriction site of pBluescript (Stratagene). They were then transferred into the *Bam*HI/*Sal*I restriction sites of the galactose-inducible yeast expression vector pYX243 (R&D Systems) as a translational fusion. The resulting constructs were introduced into *Saccharomyces cerevisiae* spermidine synthase-deficient mutant strain Y480 (MATα, *his6*, *leu2*, *ura3-52*, *spe3*Δ::*URA3*) [14]. Yeast cultures and transformation were carried out as described by Hanfrey et al. [10].

The same cDNA fragments were also transferred into the *Eco*RI restriction site of the bacterial expression vector pMAL-c2 (New England Biolabs) as a translational fusion with maltose-binding protein (MBP). The resulting constructs were introduced into *Escherichia coli* strain TB1. The recombinant proteins were induced by addition of 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to culture media for 4 h [11].

2.5. Enzyme assays

Induction of recombinant proteins and extraction of polyamines from yeast and bacterial cells were as described previously [10,11]. The dansylated polyamines were analyzed by high-performance liquid chromatography (HPLC) [15]. For feeding experiments in yeast cells, ^{14}C-labeled putrescine was added to the SD culture medium lacking leucine and glucose with 2% galactose and cells were incubated at 25°C for 12 h. For feeding experiments in bacterial cells, ^{14}C-labeled spermidine was added to the culture medium with 0.3 mM IPTG [11]. The HPLC fractions corresponding to putrescine, spermidine and spermine were collected and counted by a scintillation counter.

3. Results and discussion

3.1. Structures of the spermidine synthase-related genes in Arabidopsis

The *Arabidopsis* genome project provided us with an opportunity to search for an entire plant genome for spermidine synthase-related genes. Database searches revealed that the *Arabidopsis* genome contains four genes with a high similarity to known spermidine synthase gene sequences. These include *ACL5*, which has been shown to encode spermine synthase [11], and two genes whose mRNA sequences have been registered as *SPDS1* and *SPDS2* (accession numbers AJ251296 and AJ251297). A partial cDNA sequence of *SPDS2* has also been reported by Hashimoto et al. [16]. An additional

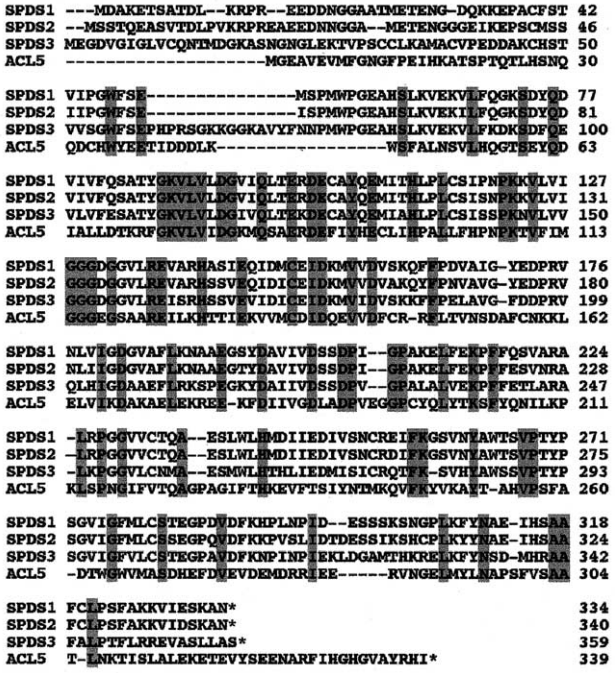

Fig. 1. Alignment of the amino acid sequences encoded by spermidine/spermine synthase-related genes in the *Arabidopsis* genome: ACL5 (AF184093), SPDS1 (AJ251296), SPDS2 (AJ251297), and SPDS3 (AY040013). Completely identical residues are shaded.

gene was found to be located on chromosome 5. Here, we tentatively name this gene *SPDS3*. The cDNA fragments containing complete coding sequences of *SPDS1*, *SPDS2* and *SPDS3* were amplified from total RNA samples prepared from young seedlings by RT-PCR with oligonucleotide primers that correspond to the putative 5′- and 3′-UTR of the respective genes. The deduced amino acid sequences of these gene products are aligned with ACL5 in Fig. 1. The calculated molecular masses of SPDS1, SPDS2, SPDS3 and ACL5 are 36.6 kDa, 37.1 kDa, 39.2 kDa and 38.5 kDa, respectively. SPDS1 and SPDS2 display 87% sequence identity with each other, 60–62% identity with SPDS3, but only about 32% with ACL5. SPDS3 shows 33% identity with ACL5. Since these protein sequences have no intercellular targeting signals, these proteins are likely to be cytoplasmic enzymes.

Fig. 2A shows the exon–intron organization of these genes aligned with related genes from other organisms. *SPDS2* contains seven introns, whose locations are also conserved within *SPDS1* and *SPDS3*, suggesting that these genes are recent derivatives from a common ancestor. *SPDS1* and *SPDS3* each have an additional splice site that interrupts the coding region corresponding to the sixth exon of *SPDS2* and is also shared by the human *SPDS* gene [17]. In addition, two exon–intron boundaries are conserved at the same position in the three *Arabidopsis* genes and the human *SPDS* gene. In contrast, the exon–intron structure of these genes is different from that of *ACL5*, which encodes a spermine synthase. Sequence relationships among spermidine and spermine synthases from various organisms were resolved using CLUSTAL X [18]. A phylogenetic tree derived from the neighbor-joining method [19] revealed that ACL5 and other known spermine synthases could be topologically separated from known spermidine synthases, with the exception of the *E. coli* spermidine synthase, which is not consistently more related to eukaryotic spermine

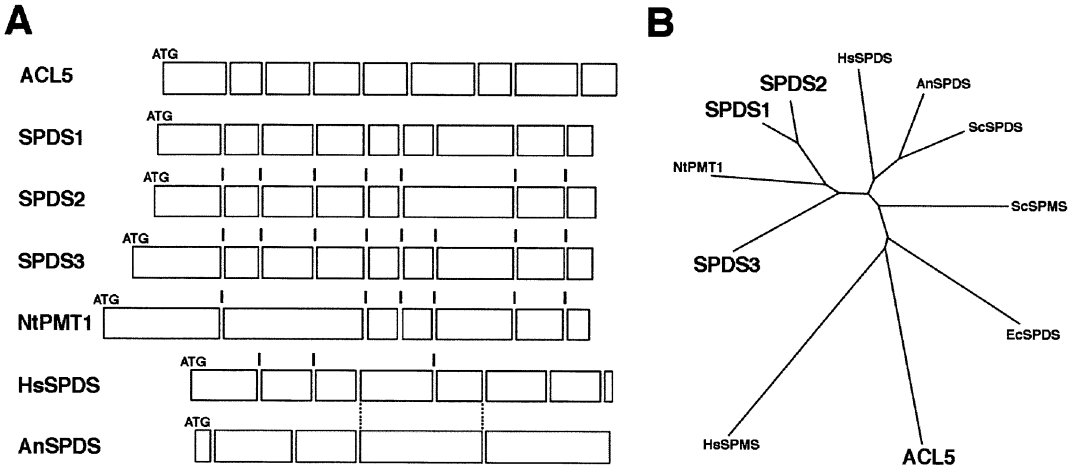

Fig. 2. Comparison of spermidine/spermine synthase-related genes from various organisms. A: Comparison of the exon–intron structures of spermidine/spermine synthase-related genes. The protein coding regions are depicted. ACL5, SPDS1, SPDS2, and SPDS3 represent *A. thaliana* genes. NtPMT1, *Nicotiana tabacum* putrescine *N*-methyltransferase (*PMT1*) gene (AF126810), HsSPDS, human spermidine synthase gene (M64231), and AnSPDS, *Aspergillus nidulans* spermidine synthase gene (AY050641). Bars indicate splicing positions equivalent to those of the *Arabidopsis SPDS1* gene. Dashed bars indicate splicing positions conserved within different organisms other than *Arabidopsis*. B: Molecular phylogenetic tree of the amino acid sequences of spermidine/spermine synthase-related proteins. The amino acid sequences were aligned using the multiple alignment program CLUSTAL X [18]. A phylogenetic tree was drawn by the neighbor-joining method using the software TREEVIEW [19]. EcSPDS, *E. coli* spermidine synthase (speE, J02804), HsSPMS, human spermine synthase (P52788), ScSPDS, *S. cerevisiae* spermidine synthase (SPE3, U27519), and ScSPMS, *S. cerevisiae* spermine synthase (SPE4, AAC19368). Other abbreviations of sequences are as indicated above.

synthases (Fig. 2B). These data suggest that spermine synthase evolved from spermidine synthase before plant and animal systems diverged. On the other hand, consistent with results of previous studies by others [16,20], the data shown in Fig. 2 suggest that putrescine *N*-methyltransferase (PMT), which catalyzes the SAM-dependent *N*-methylation of putrescine at the first committed step in the biosynthetic pathways of tropane alkaloids and nicotine in Solanaceae, evolved from plant spermidine synthase after plant and animal spermidine synthases had diverged.

3.2. Enzyme assays

In order to determine whether SPDS1, SPDS2 and SPDS3 possess spermidine synthase activity, their corresponding cDNAs were cloned into the yeast expression vector pYX243 and expressed in the yeast spermidine synthase-deficient mutant strain Y480 [14]. After the induction of the introduced genes overnight by galactose, polyamines were extracted and measured using HPLC (Fig. 3). Transformation with pYX243 alone did not allow these mutant cells to accumulate any detectable levels of spermidine. In yeast cells that produce SPDS1 or SPDS2, large amounts of spermidine accumulated, while only small amount of spermidine accumulated in cells that produce SPDS3. No detectable amount of spermidine accumulated in yeast cells that produce ACL5. We also examined the effect of the production of these proteins on the synthesis of polyamines in yeast mutant cells by adding radiolabeled putrescine to the culture media. HPLC fractions of the extract corresponding to putrescine, spermidine and spermine were collected and their radioactivities were measured. An increase in the radioactivity of the spermidine fraction was observed in all of the yeast cells that produce SPDS1, SPDS2 or SPDS3 (Table 1). These results provide compelling evidence that both *SPDS1* and *SPDS2* encode spermidine

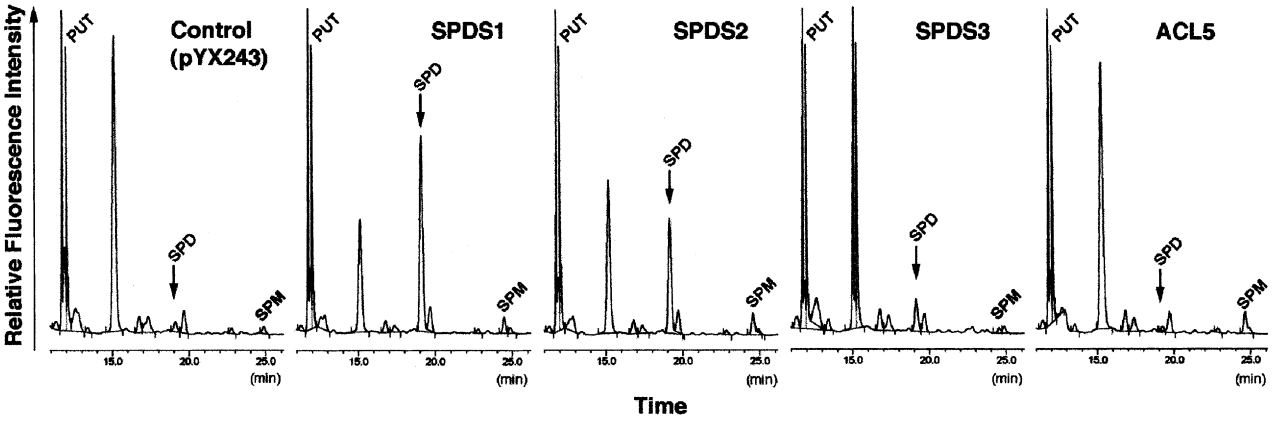

Fig. 3. Fluorescence trace of yeast polyamines as dansyl derivatives following separation by HPLC. Yeast spermidine synthase-deficient mutant cells were transformed with the expression vector pYX243 or its recombinants for producing *Arabidopsis* SPDS1, SPDS2, SPDS3 or ACL5. Fractions corresponding to putrescine, spermidine and spermine are indicated as PUT, SPD and SPM, respectively.

Table 1
Radioactivities of the HPLC fractions corresponding to putrescine, spermidine and spermine in extracts from yeast spermidine synthase-deficient mutant cells

Plasmid	Putrescine (d.p.m.)	Spermidine (d.p.m.)	Spermine (d.p.m.)
pYX243	24.0 ± 2.2	21.2 ± 1.8	20.0 ± 1.5
SPDS1/pYX243	25.0 ± 0.1	51.4 ± 1.9	17.9 ± 0.1
SPDS2/pYX243	27.3 ± 0.6	57.0 ± 9.0	19.0 ± 0.1
SPDS3/pYX243	24.0 ± 1.3	51.0 ± 7.7	18.9 ± 1.0
ACL5/pYX243	21.9 ± 0.1	20.7 ± 1.8	19.9 ± 1.4

Feeding experiments with [^{14}C]putrescine were performed twice for *S. cerevisiae* spermidine synthase-deficient mutant Y480 cells [14] carrying each construct (see Section 2).

synthase. The *SPDS3* gene product is also likely to retain spermidine synthase activity. The low amount of spermidine accumulation in yeast cells (Fig. 3) can be presumably accounted for by the requirement of certain conditions or factors for the optimal activity of the SPDS3 protein. Alternatively, it is possible that the major enzymatic function of SPDS3 is not in the synthesis of spermidine in *Arabidopsis* tissues and that it cannot be reproduced in yeast cells. The recently published crystal structure of a spermidine synthase from *Thermotoga maritima* revealed deep cavities for binding substrate and cofactor, and a loop that envelops the active site [21]. We note that the Phe residue that is invariant in all known spermidine synthases and likely contributes to the formation of a putrescine-binding cavity [21] is substituted by Val236 in SPDS3 (Fig. 1). Val236 might be important for precise positioning of the substrate for the reaction. It is unlikely that SPDS3 possesses PMT activity because 7 amino acids conserved among all available PMT sequences [22] are not conserved in SPDS3 and the amino acids at the corresponding positions in SPDS3 (Lys124, Asp125, Glu126, Asp154, Arg159, His164, and Val167) are nearly identical to those of known plant spermidine synthases.

We also performed feeding experiments using *E. coli* cells that produce these *Arabidopsis* proteins with radiolabeled spermidine. The proteins were produced as a fusion protein with MBP. Conversion of spermidine into spermine was not detected in *E. coli* cells that produce SPDS1 or SPDS2 (Table 2), indicating that these do not possess spermine synthase activity. However, *E. coli* cells that produce SPDS3 accumulated radiolabeled spermine, although its radioactivity was 10-fold lower than that of the cells that produce ACL5. Taken together with the results of yeast experiments, our data suggest that SPDS3 could have both spermidine and spermine synthase activities. However, the elucidation of the role of SPDS3 in *Arabidopsis* tissues requires further biochemical and genetic studies, including the isolation of knockout mutants of the *SPDS3* gene.

3.3. Expression analyses

The accumulation of *SPDS1*, *SPDS2*, and *SPDS3* transcripts in different organs was analyzed by RNA gel blot hybridization. Gene-specific probes for each gene detected all of these transcripts in all organs tested (Fig. 4A). While *SPDS1* and *SPDS2* transcripts were present at higher levels in roots than in other organs, *SPDS3* expression was predominant in stem internodes and flower buds as well as in roots. Our previous study revealed that *ACL5* transcript levels are also high in stem internodes, flower buds and roots ([11] and Fig. 4A). These results are consistent with the speculation that polyamines play a critical role in cell division.

Since *ACL5* expression was found to be upregulated in response to exogenous application of auxin ([11] and Fig. 4B), we examined the responses of *SPDS1*, *SPDS2* and *SPDS3* genes to plant hormone treatments. As shown in Fig. 4B, the *SPDS2* transcript level was increased by treatment of seedlings with cytokinin (100 μM kinetin) for 2 h. Cytokinin is essential for plant cell division and interacts synergistically or antagonistically with auxin to control many aspects of plant growth [23]. Taken together with the proposed functions of plant polyamines such as the control of cell division, transcription of genes, organ development and fruit ripening [3,4,7], our results suggest that cytokinin and auxin exert their effects at least in part by the production of spermidine and spermine. It has recently been reported that exogenous spermine prevents cytokinin-induced expression of *ARR5*, one of the response regulator genes in *Arabidopsis* [24]. It is tempting to speculate that the basic mechanism underlying the antagonistic interactions between cytokinin and auxin involves a balance between spermidine and spermine produced by SPDS2 and ACL5, respectively. On the other hand, the *SPDS3* transcript level was increased following abscisic acid (ABA) treatment (Fig. 4B). This suggests that ABA signaling also involves the functions of some polyamines. ABA is known to play a role in adaptation to abiotic environmental stresses including desiccation, salinity and low temperature. Considering the large body of evidence indicating that the increased resistance to these stresses is associated with the increased amounts of polyamines in plant tissues, SPDS3 might be required for ABA-mediated stress responses. In tobacco cell cultures, genes encoding ADC, ODC and *S*-adenosylmethionine decarboxylase have been shown to be upregulated by methyl jasmonate (MeJA) [25,26]. We confirmed that

Table 2
Radioactivities of the HPLC fractions corresponding to putrescine, spermidine and spermine in *E. coli* extracts

Plasmid	Putrescine (d.p.m.)	Spermidine (d.p.m.)	Spermine (d.p.m.)
pMAL-c2	91.0 ± 4.7	1311.0 ± 121.1	32.8 ± 0.3
SPDS1/pMAL-c2	98.4 ± 8.8	1779.5 ± 108.3	36.5 ± 2.1
SPDS2/pMAL-c2	107.2 ± 12.6	1558.9 ± 28.1	35.2 ± 1.3
SPDS3/pMAL-c2	104.9 ± 1.9	1628.1 ± 54.2	136.3 ± 9.3
ACL5/pMAL-c2	84.7 ± 2.3	279.7 ± 179.1	1557.2 ± 453.1

Feeding experiments with [^{14}C]spermidine were performed twice for *E. coli* TB1 cells carrying each construct (see Section 2).

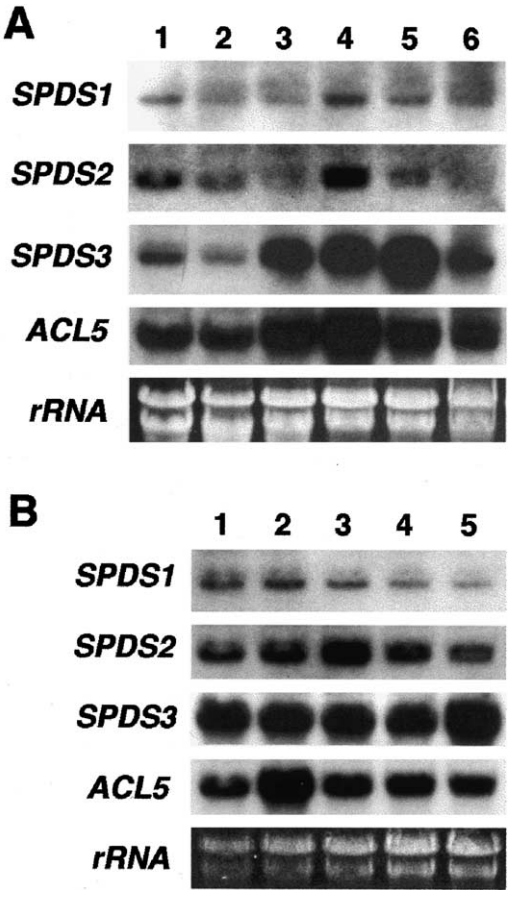

Fig. 4. Expression analyses of spermidine synthase-related genes in *Arabidopsis*. A: Organ-specific expressions of *SPDS1*, *SPDS2*, *SPDS3* and *ACL5*. RNA samples were prepared from 7-day-old whole seedlings (lane 1), leaves (lane 2), stem internodes (lane 3), roots (lane 4), inflorescences (lane 5), and siliques (lane 6) of adult flowering plants of the wild type. B: Effects of plant hormones on the expressions of *SPDS1*, *SPDS2*, *SPDS3* and *ACL5*. Seven-day-old seedlings grown in liquid MS media were treated for 2 h with mock (lane 1), 3-indoleacetic acid (lane 2), 6-benzylaminopurine (lane 3), gibberellin A3 (lane 4), and ABA (lane 5). Final concentrations of these hormones were 100 μM. In A and B, total RNA (10 μg per lane) was loaded and transferred to GeneScreen. Ethidium bromide staining of the agarose gels prior to blotting was done to confirm integrity of the RNA and loading of a similar amount of *rRNA*.

treatment of *Arabidopsis* seedlings for 2 h with 100 μM of MeJA, salicylic acid, brassinosteroid, or 1-aminocyclopropane-1-carboxylic acid, a precursor of ethylene, causes no alterations in the transcript levels of *SPDS1*, *SPDS2*, *SPDS3* and *ACL5* (data not shown).

In summary, we have shown that at least two of the three spermidine synthase-related genes in the *Arabidopsis* genome, *SPDS1* and *SPDS2*, encode spermidine synthase that is functional in non-native systems. In a future study, knockout mutants of these genes must be isolated to elucidate the modes of actions of polyamines produced by them in plant cells.

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