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Thermospermine is Required for Stem Elongation in *Arabidopsis thaliana*

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Loss-of-function mutants of the *ACAULIS5* (*ACL5*) gene in *Arabidopsis thaliana* have severe defects in stem elongation. *ACL5* was previously reported as encoding a spermine synthase. A more recent study, however, showed that the bacterial expressed recombinant ACL5 protein catalyzes the conversion of spermidine to thermospermine, a structural isomer of spermine, rather than to spermine. In the present study, we found that thermospermine was detected in wild-type seedlings but was not detectable in the *acl5-1* mutant. We further examined the effect of exogenous application of these isomers on the growth of *acl5-1*. Daily application of 0.1 mM thermospermine onto the shoot apex partially rescued the dwarf phenotype of *acl5-1*, while that of spermine had no effects on the morphology of the mutant. The *acl5-1* transcript level in *acl5-1* seedlings, which is much higher than the *ACL5* transcript level in wild-type seedlings, was reduced by exogenous thermospermine. Thus we conclude that thermospermine is indeed produced through the action of ACL5 and required for stem elongation in Arabidopsis.

Keywords: *acaulis5* — *Arabidopsis thaliana* — Polyamine — Spermine — Stem elongation — Thermospermine.

Abbreviations: ACL5, ACAULIS5; SPDS, SPERMIDINE SYNTHASE; SPMS, SPERMINE SYNTHASE; SAC51, SUPPRESSOR OF ACAULIS5-1; MS, Murashige-Skoog; AMV, Avian myeloblastosis virus; GUS, β-glucuronidase; PAO, polyamine oxidase; uORF, upstream open reading frame; UTR, untranslated region

Introduction

Polyamines are ubiquitous low-molecular-mass polycations involved in a wide range of cellular processes, including chromatin condensation, maintenance of DNA structure, RNA processing, regulation of translation, modulation of enzyme activities, and stabilization of membranes (Pegg 1988, Cohen 1998). The biosynthesis of three major polyamines, putrescine (1,4-diaminobutane), spermidine, and spermine, proceeds from the amino acid arginine. Sequential alkylation of amino groups of putrescine with decarboxylated S-adenosylmethionine 3-aminopropyl donor yields spermidine and spermine. In plants, polyamines frequently exert effects resembling those of some plant hormones and appear to function in stimulation of cell division, fruit ripening, and stress signaling (Kumar et al. 1997, Walters 2003, Kusano et al. 2007). Previously, we reported that double mutants of *SPDS1* and *SPDS2*, both of which encode spermidine synthase in *Arabidopsis thaliana*, are embryonic lethal and spermidine is essential for survival of plants (Imai et al. 2004a). The Arabidopsis genome also has two genes identified as encoding spermine synthase, *ACAULIS5* (*ACL5*) (Hanzawa et al. 2000) and *SPMS* (Panicot et al. 2002). Loss-of-function mutants of *ACL5* show a severe dwarf phenotype (Hanzawa et al. 2000), while those of *SPMS* show wild-type phenotype (Imai et al. 2004b). *spms acl5* double mutants show dwarf phenotype due to the *acl5* mutation, suggesting that ACL5 and SPMS may function in spatially and temporally distinct manners (Imai et al. 2004b). However, these studies have overlooked the difference between spermine and its structural isomer, thermospermine, which cannot be distinguished from each other under HPLC conditions. Only recently, Knott et al. (2007) identified a gene homologous to *ACL5* from the diatom *Thalassiosira pseudonana*. They have shown that the bacterial expressed recombinant ACL5 proteins from Arabidopsis and the diatom produce thermospermine rather than spermine.

Thermospermine was first identified from the thermophilic bacterium *Thermus thermophilus* (Oshima 1979) and could also be detected in higher plants such as pea (Hamana and Matsuzaki 1985) and alfalfa (Bagga et al. 1997). However, its biological function has remained almost uninvestigated. In this study, we found that thermospermine was not detected in the extract of *acl5-1* plants and its exogenous application partially rescued the dwarf phenotype of *acl5-1*. Our results provide in vivo evidence that thermospermine is produced through the action of ACL5 and required for stem elongation in Arabidopsis.

Results

Detection of thermospermine in plant extracts by thin-layer chromatography

To examine whether or not ACL5 mediates the production of thermospermine in planta, we prepared

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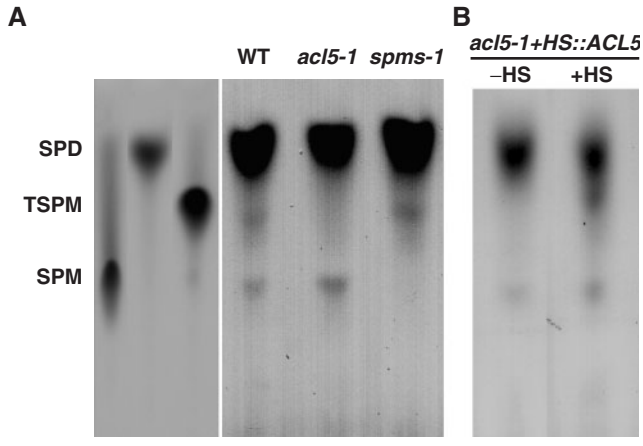

Fig. 1 Detection of thermospermine in *Arabidopsis* extracts. (A) Thin-layer chromatogram (TLC) of polyamines extracted from wild-type (WT), *acl5-1*, and *spms-1*seedlings. All seedlings were grown for 3 d under complete darkness in the presence of 0.1 mM spermidine. Synthetic spermidine (SPD), thermospermine (TSPM), and spermine (SPM) are shown as reference in the left panel. (B) TLC of polyamines extracted from transgenic *acl5-1* seedlings carrying the *HS::ACL5* fusion construct. Seedlings were grown for 3 d under complete darkness in the absence of spermidine and polyamines were extracted before (–HS) or after (+HS) heat-shock treatment.

polyamine extracts from wild-type and *acl5-1* seedlings. Instead of a standard protocol to detect common polyamines by dansylation followed by HPLC, thin-layer chromatography (TLC)with a solvent system containing formaldehyde (Shirahata et al. 1983) was used to separate spermine and thermospermine. However, we could not detect thermospermine in the extract of wild-type seedlings grown in normal MS medium by TLC and ninhydrin visualization. Therefore, the seedlings were supplied with 0.1 mM spermidine for 3 d before analysis. As shown in Fig. 1A, wild-type seedlings accumulated both spermine and thermospermine while *acl5-1* seedlings contained spermine but no detectable thermospermine. We also confirmed that the extract of *spms-1* seedlings contained thermospermine but no detectable spermine.

We have previously shown that expression of the *ACL5* cDNA under the control of a heat shock gene promoter restores the dwarf phenotype of *acl5-1* transgenic plants in a heat shock-dependent manner (Hanzawa et al. 2000). We therefore examined whether *acl5-1* transgenic seedlings carrying the *HS::ACL5* fusion construct produce thermospermine or not. The seedlings were grown in normal MS medium for 3 d and polyamines were extracted before or after heat shock treatment at 37°C for 1 h. TLC analysis revealed that thermospermine was detectable in the seedlings after heat shock induction but not in the seedlings before treatment (Fig. 1B). *acl5-1* seedlings with no transgene were also confirmed to contain no thermospermine after heat shock (data not shown).

Recovery of the growth of acl5-1 by exogenous thermospermine

To test whether or not exogenous thermospermine can rescue the dwarf phenotype of *acl5-1* plants, we performed feeding experiments with thermospermine. When *acl5-1* mutants were germinated, grown on rockwool supplemented with MS salt solution, and fed daily with 20 µl of 0.1 mM thermospermine as drops on their shoot apices, they showed partial but significant recovery of the stem growth, while *acl5-1* plants fed with mock (water) or with spermine showed no recovery of the stem growth (Fig. 2A–D). When shoot apices of wild-type plants were treated daily with 20 µl of mock or 0.1 mM thermospermine, there was no difference between the heights of both plants (Fig. 2E–G). Daily feeding with 1 ml of 0.1 mM thermospermine on root tissues of *acl5-1* mutants resulted in no recovery of the mutant phenotype (data not shown).

Negative feedback regulation of ACL5 by thermospermine

Our previous study revealed that the *acl5-1* transcript level in *acl5-1* plants was much higher than the *ACL5* transcript level in wild-type plants, suggesting of a negative feedback regulation of *ACL5* expression by its reaction product (Hanzawa et al. 2000). We therefore examined the effect of exogenous thermospermine on the expression of *ACL5*. Thermospermine was added to the liquid MS medium. Quantitative reverse transcription-PCR experiments revealed that the level of the *acl5-1* transcript which encodes an inactive enzyme in *acl5-1* seedlings was drastically reduced and reached to the wild-type level after 24 h of thermospermine treatment, while the level of the *ACL5* transcript in wild-type seedlings was also slightly reduced (Fig. 3). We also confirmed that the level of the *acl5-1* transcript was not altered by spermine treatment of *acl5-1* seedlings (Fig. 3). On the other hand, the level of the *SPMS* transcript which is normal in *acl5-1* seedlings (Imai et al. 2004b) was not altered by exogenous thermospermine (data not shown).

Effects of exogenous thermospermine on gene expression

We have previously identified genes showing altered expression in *acl5-1* plants. These include *ENDOXYLOGLUCAN TRANSFERASE-A1* (*EXGT-A1*), which is implicated in regulating cell-wall extensibility (Akamatsu et al. 1999) and members of the HD-Zip III homeobox gene family, which are required for vascular development and the control of leaf polarity (Prigge et al. 2005). Expression of *EXGT-A1* is reduced in *acl5-1* seedlings grown in MS agar plates (Hanzawa et al. 1997), while that of HD-Zip III genes, *ATHB1* and *PHABULOSA* (*PHB*) is increased in the mutant (Imai et al. 2006). We therefore examined the effect of exogenous application of thermospermine on the expression of these genes. As shown

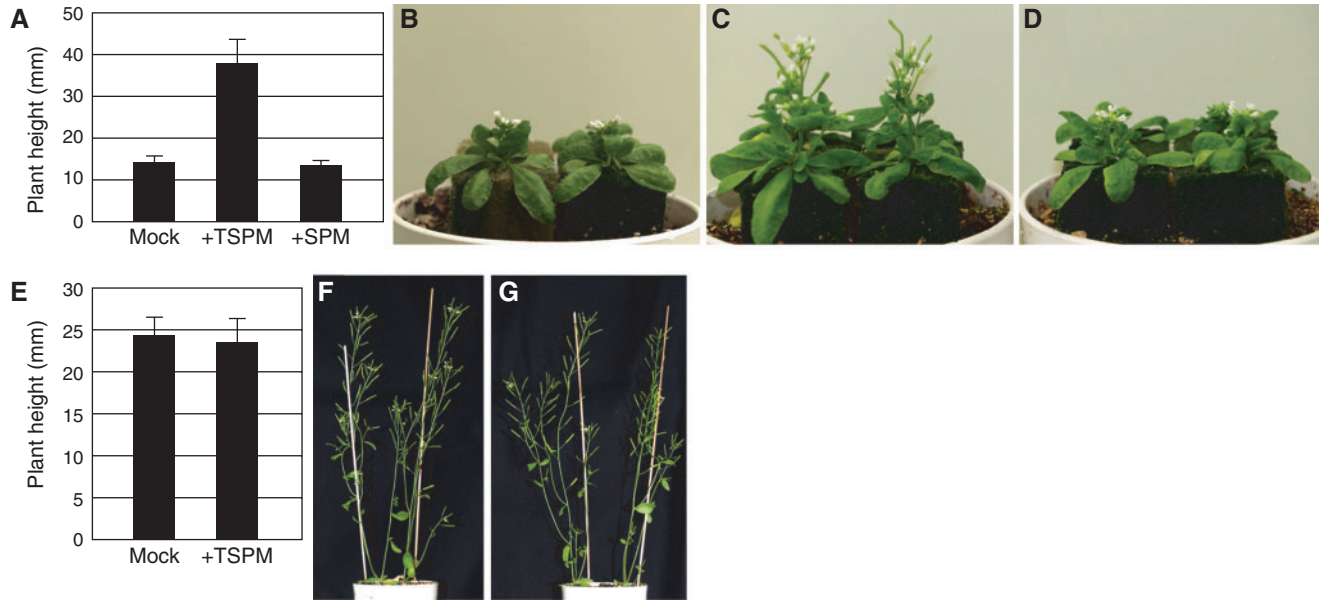

Fig. 2 Recovery of the phenotype in *acl5-1* plants by exogenous thermospermine. (A) Height of 30-d-old flowering plants of *acl5-1*. Twenty µl of mock (water), 0.1 mM thermospermine (+TSPM) or spermine (+SPM) was added daily to the shoot apex. Values are averages of eight individuals for each treatment. (B) Phenotype of mock-treated 30-d-old *acl5-1* plants. (C) Phenotype of 30-d-old *acl5-1* plants treated daily with thermospermine. (D) Phenotype of 30-d-old *acl5-1* plants treated daily with spermine. (E) Height of 30-d-old flowering plants of wild-type L*er*. Plants were treated as described above. (F) Phenotype of mock-treated 30-d-old wild-type plants. (G) Phenotype of 30-d-old wild-type plants treated daily with thermospermine.

in Fig. 4A, in contrast to the case with the seedlings grown in MS agar plates, *acl5-1* seedlings grown in liquid MS medium showed a slightly higher expression level of *EXGT-A1* compared with the wild type before thermospermine treatment. The transcript level of *EXGT-A1* was up-regulated by thermospermine in both *acl5-1* and wild-type seedlings, while transcript levels of *ATHB8* and *PHB*, two members of the HD-Zip III family, were down-regulated in response to thermospermine in both seedlings (Fig. 4). The degree of change in transcript levels for these genes was more prominent in *acl5-1* than in the wild type. The transcript levels of these genes remained unaltered after 24 h of spermine treatment in *acl5-1* and wild-type seedlings (data not shown).

sac51-d is a dominant suppressor mutant of *acl5-1* and completely rescues its dwarf phenotype (Imai et al. 2006). *SAC51* contains five upstream open reading frames (uORFs) followed by a sequence encoding a basic helix-loop-helix (bHLH) transcription factor. Because the *sac51-d* allele contains a point mutation in the 4th uORF that introduces a premature termination codon and leads to an increase in the transcription and translation of *SAC51*, suppression of the mutant phenotype in *sac51-d acl5-1* may be attributed to the overproduction of the SAC51 bHLH protein. However, the relation between thermospermine and *SAC51* expression has remained unknown. We examined the effect of thermospermine on the expression of

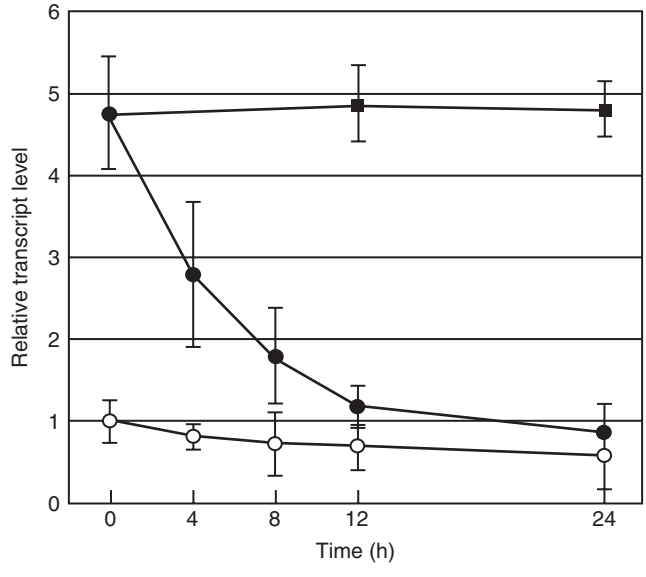

Fig. 3 Effect of thermospermine and spermine on transcript levels of *ACL5*. Total RNA was prepared from wild-type and *acl5-1* seedlings, which were grown for 7 d in liquid MS media and incubated with 0.1 mM thermospermine or spermine for the indicated hours. Transcript levels of *ACL5* in the wild type incubated with thermospermine (open circles), *acl5-1* in the mutant incubated with thermospermine (closed circles), and *acl5-1* in the mutant incubated with spermine (closed squares), were quantified by quantitative real-time PCR and relative values were normalized to the expression of *ACTIN8*. The basal level of the untreated wild-type sample was set at 1.0. Values are averages of three measurements.

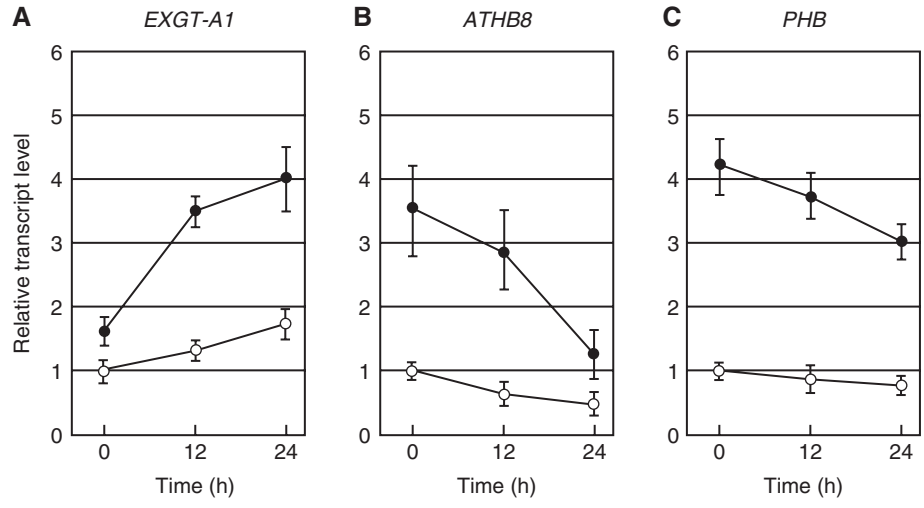

Fig. 4 Effect of thermospermine on transcript levels of *EXGT-A1*, *ATHB8*, and *PHB*. Preparation of total RNA and quantification of transcript levels of *EXGT-A1* (A), *ATHB8* (B), and *PHB* (C) in the wild type (open circles) and the *acl5-1* mutant (closed circles) were performed as described in the legend of Fig. 3. The basal level of the untreated wild-type sample was set at 1.0 for each gene. Values are averages of three measurements.

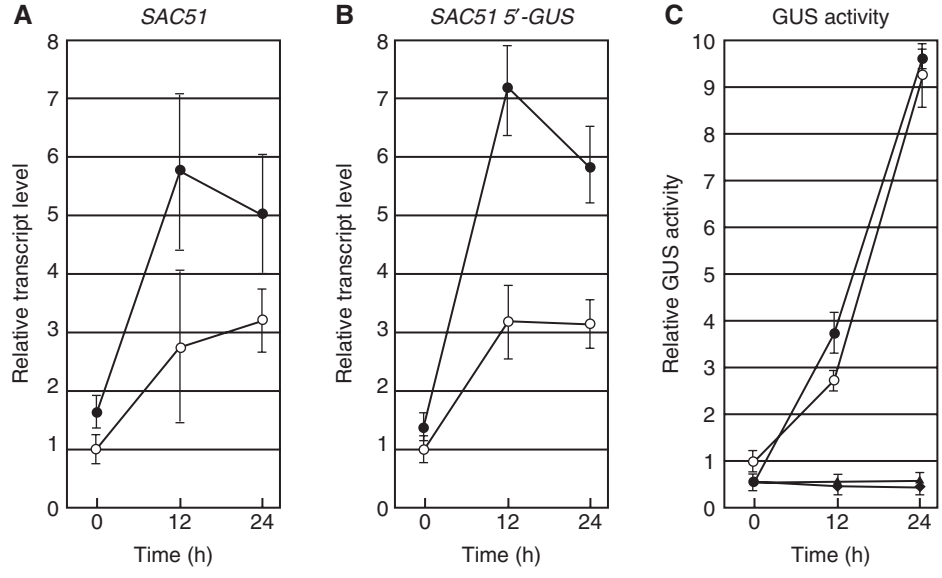

Fig. 5 Effect of thermospermine on the expression of *SAC51*. Preparation of total RNA and quantification of transcript levels of *SAC51* (A) and *GUS* (B) were performed for transgenic line 1 carrying the *SAC51-GUS* fusion construct (Imai et al. 2006) of the wild-type background (open circles) and that of the *acl5-1* background (closed circles), as described in the legend of Fig. 3. GUS activity (C) was measured for the *SAC51-GUS* line #1 of the wild type treated with 0.1 mM thermospermine (open circles) and that of *acl5-1* treated with 0.1 mM thermospermine (closed circles), 40 µg/ml actinomycin D (closed triangles), and 40 µg/ml actinomycin D plus 0.1 mM thermospermine (closed diamonds). In the latter experiments, actinomycin D was added to the media 1 h before time 0. The basal level or activity of the untreated wild-type sample was set at 1.0 in each panel. Values are averages of three measurements.

SAC51 and found that *SAC51* was up-regulated by thermospermine in both *acl5-1* and wild-type seedlings (Fig. 5A). We also confirmed that *SAC51* expression was not altered by exogenous spermine in both seedlings (data not shown). *SAC51* expression was further examined by using the *GUS* reporter gene fused to a *SAC51* promoter region and its 5' leader sequence in transgenic plants. As was the transcript level of endogenous *SAC51*, the GUS transcript level was increased by thermospermine (Fig. 5B). The degree of change in these transcript levels was higher in *acl5-1* than in the wild type. On the other hand, as reported for the seedlings grown in MS agar plates (Imai et al. 2006),

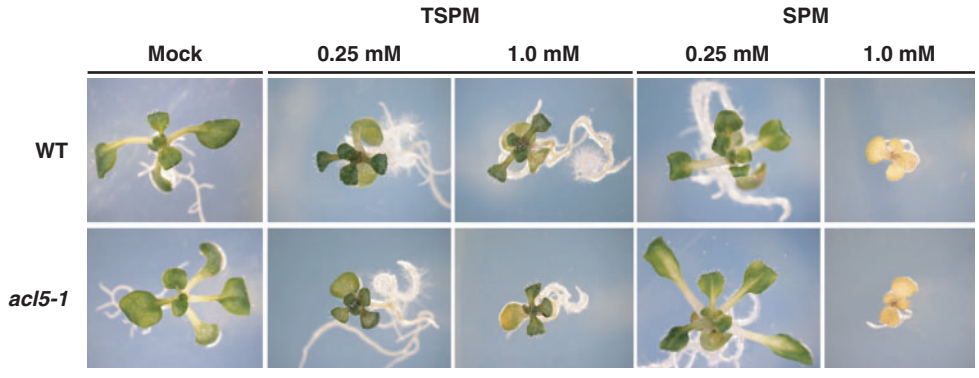

Fig. 6 Inhibitory effects of high concentrations of therspermine and spermine on plant growth. Wild-type (WT) and *acl5-1* seedlings were grown for 7 d on MS agar plates containing the indicated concentrations of thermospermine (TSPM) or spermine (SPM) under continuous light at 22°C.

GUS activity was significantly lower in *acl5-1* than in the wild type before treatment (Fig. 5C), suggesting reduced translation of *SAC51* in the absence of thermospermine. The GUS activity was increased after thermospermine treatment along with the increase in *SAC51* and *GUS* transcript levels and reached to the same level in both wild-type and *acl5-1* seedlings (Fig. 5C). Considering the magnitude of the increase in these transcript levels, however, translation of the *SAC51-GUS* transcript did not seem to be more accelerated in *acl5-1* than in the wild type by thermospermine. Thus, to examine further the effect of thermospermine on *SAC51-GUS* translation in *acl5-1* seedlings, the transcriptional inhibitor actinomycin D (40 μg/ml) was added with or without thermospermine to the medium. The GUS activity remained unchanged after 24 h of these treatments and we observed no acceleration of the *SAC51-GUS* translation attributable to thermospermine (Fig. 5C). These results were reproduced in three independent transgenic lines carrying the same construct.

Toxic effects of thermospermine on the growth of seedlings

We finally investigated the growth of wild-type and *acl5-1* seedlings in the presence of an excess amount of exogenous thermospermine or spermine. Seedlings were grown for 7 d on MS agar plates supplemented with increasing concentrations of these polyamines. When 0.25–1 mM thermospermine was added, an inhibition of leaf expansion was observed in both wild-type and *acl5-1* seedlings (Fig. 6). On the other hand, 0.25 mM spermine resulted in no change in the growth of wild-type and *acl5-1* seedlings, and 0.5–1 mM spermine led to a progressive chlorosis and inhibition of leaf expansion in both seedlings (Fig. 6). Higher than 1 mM concentration of thermospermine or spermine led to chlorosis and a progressive inhibition of seed germination (data not shown). Germination of wild-type and *acl5-1* seeds was completely inhibited by 5 mM thermospermine or spermine. Conclusively, we found no difference between wild type and *acl5-1* with respect to responses to high concentrations of thermospermine and spermine.

Discussion

Spermine and thermospermine levels in living organisms have usually been examined as mixtures, and their structural and functional differences have rarely been addressed in past work, while thermospermine was recently shown to be produced in vitro by the recombinant ACL5 protein (Knott et al. 2007). Here, by using TLC followed by ninhydrin staining, we separately detected spermine and thermospermine in extracts from Arabidopsis seedlings that were fed with spermidine as substrate. Although we have not quantitated the amounts of these isomers, wild-type seedlings may have thermospermine to a lesser extent than spermine because we could not detect thermospermine in wild-type seedlings grown in the absence of exogenous spermidine. We further found that transgenic *acl5-1* plants carrying the *HS::ACL5* fusion construct produced detectable levels of thermospermine after heat shock induction with no exogenous supply of spermidine. Based on these results, we conclude that *ACL5* encodes thermospermine synthase, while *SPMS* encodes spermine synthase. Fig. 7 shows a revised summary of polyamine biosynthetic pathways in Arabidopsis. Thermospermine has been suggested to be converted further into homocaldopentamine [$H_2N(CH_2)_3NH_2(CH_2)_3NH_2(CH_2)_3NH_2(CH_2)_4NH_2$] and homocaldohexamine [$H_2N(CH_2)_3NH_2(CH_2)_3NH_2(CH_2)_3NH_2(CH_2)_3NH_2(CH_2)_4NH_2$] in alfalfa (Bagga et al. 1997). We could not detect these uncommon polyamines in wild-type Arabidopsis seedlings under our TLC conditions. The Arabidopsis genome contains no additional genes with high similarity to *ACL5*, *SPMS*, *SPDS1*, and *SPDS2* (Hanzawa et al. 2002, Panicot et al. 2002). However, because thermospermine, homocaldopentamine, and homocaldohexamine

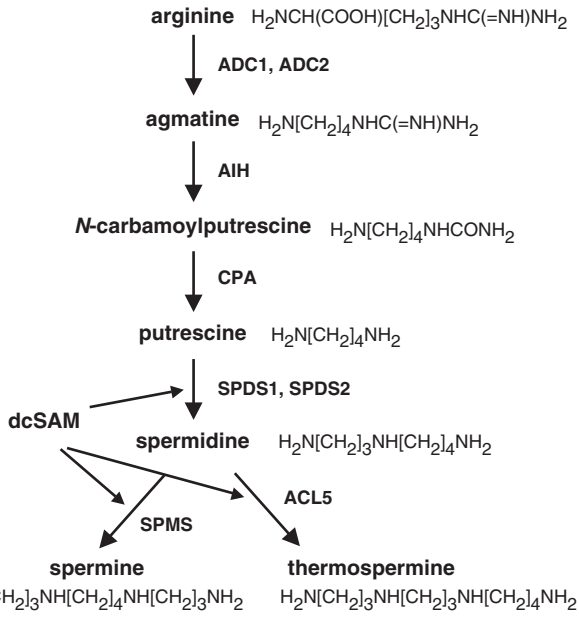

Fig. 7 Biosynthetic pathway of polyamines in Arabidopsis. All names of the gene products that catalyze the respective reaction and are assigned in the Arabidopsis genome are indicated. ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; dcSAM, decarboxylated *S*-adonosylmethionine.

are successively formed by the addition of a propylamine group donated by decarboxylated *S*-adenosylmethionine, to spermidine on the same side of the butyl group as the previous propylamine groups, we cannot rule out the possibility that ACL5 possesses a broad substrate specificity and mediates the production of homocaldopentamine and homocaldohexamine.

To the best of our knowledge, the effect of thermospermine on plant growth has not been reported so far. We revealed that exogenous application of thermospermine to shoot apices resulted in a partial but significant recovery from the dwarf phenotype in *acl5-1*. When applied to the root tissue, thermospermine did not rescue the phenotype under our experimental conditions. It is less likely, however, that thermospermine cannot be translocated from root to shoot. Spermidine has been shown to be absorbed through roots and translocated in part to shoots in Arabidopsis (Tassoni et al. 2000). Because even shoot-applied thermospermine did not fully rescue the mutant phenotype, the optimal condition of exogenous thermospermine treatment for recovery of the stem growth in *acl5-1* might be found by further investigation. While polyamine transporters have been suggested to be present in the plasma membrane and tonoplast of Arabidopsis cells (Colombo et al. 1992), polyamines are known to be metabolized by a cell wall-localized polyamine oxidase (PAO) in plants (Sebela et al. 2001). This reaction causes the production of hydrogen peroxide, which has been implicated in lignification and stiffening of cell walls as well as defense signaling (Cona et al. 2006). These outcomes would rather have a negative effect on cell elongation, as exogenous spermidine confers plants with shorter stalks in Arabidopsis (Tassoni et al. 2000). Exogenous thermospermine might also be oxidized, at least in part, by PAO and negatively affect the stem growth, in contrast to intracellular thermospermine whose predicted function is to promote cell elongation. Excess concentrations of thermospermine and spermine had inhibitory effects on leaf expansion, chlorophyll synthesis, and seed germination, although the effects of thermospermine appeared to be slightly different from those of spermine with respect to the concentration (Fig. 6). Metabolism of exogenous thermospermine and spermine including degradation and conjugation, and the mechanism by which they are incorporated into cells need to be further investigated in future studies.

We found that the *ACL5* transcript level was indeed under negative feedback control by thermospermine, suggesting a requirement for strict control of the endogenous thermospermine level. This is in contrast to the *SPMS* transcript level, which is neither altered in the *spms-1* mutant nor apparently down-regulated by spermine treatment (Imai et al. 2004b). *SPMS* has been suggested to play a protective role against high salt and drought stresses (Yamaguchi et al. 2006, 2007). The lack of feedback control of *SPMS* transcription might be related to the nonrequirement of spermine under normal growth conditions. It is also possible that *SPMS* expression is regulated post-transcriptionally by spermine. In any case, our results indicate that the regulation of *ACL5* expression is independent of that of *SPMS* expression. A *cis*-regulatory sequence named as polyamine-responsive element and its trans-acting factor are known to be involved in regulating polyamine-dependent gene expression in mammals (Wang et al. 1998) but remain unidentified in plants. The *ACL5* promoter could provide a useful tool for unraveling the mechanisms responsible for thermospermine-dependent repression of gene expression.

Exogenous thermospermine was also found to down-regulate the expression of the HD-Zip III homeobox genes, which is increased in *acl5-1* (Imai et al. 2006). The dwarf phenotype of *acl5-1* always accompanies the overproliferation of lignified vascular cells in the stem (Hanzawa et al. 1997) and another allele of the *ACL5* locus has been identified as the *thickvein* (*tkv*) mutant (Clay and Nelson 2005). Because HD-Zip III genes are known to promote vascular development (Baima et al. 2001, Prigge et al. 2005), derepression of their expression by thermospermine deficiency might cause abnormal lignification of cells and inhibit their elongation in *acl5*/*tkv* mutants. It will also be interesting to determine whether expression of HD-Zip III

genes are directly repressed by thermospermine or thermospermine-dependent transcription repressors. One candidate for such repressors might be *SAC51* whose expression was shown to be up-regulated by thermospermine (Fig. 5), although it remains to be determined whether the SAC51 bHLH protein acts as a transcription activator or repressor. We previously revealed that the translational efficiency of the GUS transcript fused to the *SAC51* 5' leader sequence containing all five uORFs was significantly reduced in *acl5-1*, suggesting the requirement for *ACL5* in the acceleration of *SAC51* translation (Imai et al. 2006). However, the experiments using actinomycin D suggested no enhancement of the *SAC51-GUS* translation by exogenous thermospermine (Fig. 5C). One possibility for the function of thermospermine is that it plays a role in transcription-associated modifications of certain genes including *SAC51* that accelerate their translation. We also note that, in addition to the presence of uORFs, the *SAC51* transcript contains a long 3'-untranslated region (UTR) of 550 bp. Long 3'-UTRs are characteristic of highly regulated genes and may be important in regulating mRNA stability, localization, and translational efficiency (Wickens et al. 2002, Kuersten and Goodwin 2003, Amrani 2004, Kozak 2004). Detailed studies using a transgenic reporter and cell-free in vitro transcription/translation systems are underway and will provide insights into the action of thermospermine in the *SAC51* expression.

In conclusion, this study provides firm evidence that thermospermine, but not spermine, plays a critical role in stem elongation in Arabidopsis. Thermospermine is also detected in insects and spiders (Hamana et al. 2004), although the genes orthologous to *ACL5* have not yet been identified in animal genomes. Taking into consideration its wide distribution in nature, it is likely that thermospermine plays nonessential but potentially beneficial roles in fundamental cellular processes common to all organisms. Such roles could have become associated with the control of plant forms in the evolution of higher plants. Like a variety of growth responses to exogenous phytohormones in plants, the responses to exogenous thermospermine may provide a useful assay system for genetic and physiological studies of plant development.

Materials and Methods

Chemicals

All polyamines were used as hydrochloride salts. Spermidine and spermine were purchased from Sigma (St. Luis, MO, USA). Thermospermine was synthesized by the published method (Niitsu et al. 1992). Murashige-Skoog (MS) salts for plant nutrition were purchased from Wako (Osaka, Japan).

Plant material and growth conditions

Arabidopsis thaliana ecotype Landsberg *erecta* (L*er*) was used as the wild type. *acl5-1* and *spms-1* mutants were as described (Hanzawa et al. 2000, Imai et al. 2004b). A transgenic *acl5-1* line carrying the full-length *ACL5* cDNA fused with a heat-shock gene promoter was as described (Hanzawa et al. 2000). Transgenic lines carrying the β-glucuronidase (GUS) reporter gene fused with a *SAC51* promoter and its 5′ leader sequence were as described (Imai et al. 2006). Unless otherwise stated, all plants were grown under continuous fluorescent light at 22°C. For gene expression analyses, seeds were germinated, aerobically cultivated by shaking in liquid MS media containing 3% sucrose for 7 d, and applied with 0.1 mM thermospermine or spermine. For polyamine extraction, seeds were germinated and grown under complete darkness for 3 d on MS plates containing 0.8% agar, 3% sucrose, and 0.1 mM or no spermidine.

Real-time PCR assay

Total RNA was isolated as described (Imai et al. 2004b). For each sample, 1 μg of total RNA was reverse transcribed to cDNA using AMV reverse transcriptase according to the accompanying protocol (Takara, Kyoto, Japan). Real-time PCR was performed in a DNA Engine Opticon2 System (Bio-Rad, Hercules, CA, USA) using the iQ SYBR Green Supermix (Bio-Rad) and gene-specific primers (Imai et al. 2006). Transcript levels of *ACTIN8* were used as a reference for normalization.

Thin-layer chromatography

0.5 g fresh weight of 3-d-old etiolated seedlings were ground in liquid nitrogen and suspended in 200 μl of 5% (w/v) perchloric acid. After centrifugation, the supernatant was neutralized with 1.5 N KOH and settled on ice for 20 min. After centrifugation, the supernatant was mixed with an equal volume of 25 mM sodium acetate buffer pH 5.5 and applied to a low-capacity cation-exchange column, Vivapure C Mini M (Sartorius, Göttingen, Germany). The column was washed twice with 25 mM sodium acetate buffer. Polyamines were eluted with 50 μl of 1 M NaCl in the sodium acetate buffer and analyzed by TLC in a solvent system of *n*-butanol:acetic acid:pyridine:37% formaldehyde (3:3:2:1) (Shirahata et al. 1983). Polyamines on TLC plates (Silica gel 60F-254; Merck, Darmstadt, Germany), were detected with ninhydrin spray.

GUS assays

GUS activity was quantified using the fluorometric 4-methylumbelliferyl-β-D-glucuronide assay (Jefferson et al. 1987). Fluorescence was measured with a spectrofluorophotometer RF-1500 (Shimadzu, Kyoto, Japan) at λ355/λ460. Protein content was determined using the Bradford assay (Bio-Rad).

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