Thermospermine is Not a Minor Polyamine in the Plant Kingdom

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Thermospermine is a structural isomer of spermine, which is one of the polyamines studied extensively in the past, and is produced from spermidine by the action of thermospermine synthase encoded by a gene named ACAULISS (ACL5) in plants. According to recent genome sequencing analyses, ACL5-like genes are widely distributed throughout the plant kingdom. In Arabidopsis, ACL5 is expressed specifically during xylem formation from procambial cells to differentiating xylem vessels. Loss-of-function mutants of ACL5 display overproliferation of xylem vessels along with severe dwarfism, suggesting that thermospermine plays a role in the repression of xylem differentiation. Studies of suppressor mutants of acl5 that recover the wild-type phenotype in the absence of thermospermine suggest that thermospermine acts on the translation of specific mRNAs containing upstream open reading frames (uORFs). Thermospermine is a novel type of plant growth regulator and may also serve in the control of wood biomass production.

Keywords: ACL5 • Arabidopsis thaliana • Polyamine • Thermospermine • uORF.

Abbreviations: ACL5, ACAULISS; ADC, arginine decarboxylase; AdoMetDC/SAMDC, S-adenosylmethionine decarboxylase; bHLH, basic helix–loop–helix; BUD2, BUSHY AND DWARF2; dcAdoMet/dcSAM, decarboxylated S-adenosylmethionine; elf5A, eukaryotic initiation factor 5A; GABA, γ-aminobutyric acid; MP, MONOPTEROS; MTA, S-methylthioadenosine; MTN, MTA nucleosidase; ODC, ornithine decarboxylase; ORF, open reading frame; PAO, polyamine oxidase; PMT, putrescine N-methyltransferase; SAC, SUPPRESSOR OF ACL5; SPDS, spermidine synthase; SPMS, spermine synthase; TAAPT, triamine/aminopropyltransferase; TKV, THICKVEIN; TGase, transglutaminase; TSPMS, thermospermine synthase; uORF, upstream open reading frame.

Introduction

Polyamines are positively charged small organic compounds found in all living cells and play versatile roles in regulating fundamental cellular processes such as protein synthesis and post-translational modification (Tabor and Tabor 1999, Igarashi and Kashiwagi 2000, Wallace et al. 2003). One of the major polyamines, spermine, was discovered as a crystal in human semen by Van Leeuwenhoek in 1678 and named after its origin in the late 19th century. The crystal has now been identified as spermine phosphate. There are two pathways for the biosynthesis of the diamine putrescine from arginine, one via ornithine and the other via agmatine (Fig. 1). In the former pathway, arginine is converted to ornithine by arginase. Then, ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to form putrescine. The ODC pathway is dominant in animals and fungi, and the ODC reaction is the first and rate-limiting step in polyamine biosynthesis. In the latter, arginine is decarboxylated by arginine decarboxylase (ADC) to agmatine, which is then hydrolyzed by agmatine ureohydrolase (agmatinase) or by a combination of agmatine iminohydrolase and N-carbamoylputrescine amidohydrolase to form putrescine. The ADC pathway may be the main route for polyamine biosynthesis in some bacteria and plants. Indeed, the genes encoding ODC are absent in the genomes of some plant species including Arabidopsis thaliana (Hanfrey et al. 2001). Putrescine is then successively converted to the triamine spermidine and the tetramine spermine by spermidine synthase (SPDS) and spermine synthase (SPMS), respectively (Fig. 1). These reactions involve the addition of aminopropyl groups supplied from decarboxylated S-adenosylmethionine (dcAdoMet/dcSAM) that is converted from AdoMet/SAM by AdoMet/SAM decarboxylase (AdoMetDC/SAMDC). In addition to these three major polyamines, putrescine, spermidine and spermine, which are widely distributed in both prokaryotes and eukaryotes, certain uncommon polyamines have been found in some organisms. Extremely thermophilic bacteria and archaea are known to contain long-chain polyamines and branched polyamines, suggesting that these polyamines are important for life at temperature extremes (Oshima 2007). In higher plants, norspermidine and norspermine have been detected in alfalfa and cotton (Rodriguez-Garay et al. 1989, Kuehn et al. 1990). The diamine cadaverine, which is formed from lysine by lysine decarboxylase, may be required for the root growth of soybean seedlings (Gamarnik and Frydman 1991). However, while physiological functions or effects of the three major polyamines have been...
extensively studied in the past, those of minor cellular polyamines remain to be explored in depth.

Recent molecular and genetic approaches have revealed that polyamines play critical roles in growth and development of higher plants. Studies on the Arabidopsis ADC2 gene indicate that putrescine plays a role in drought and wounding responses (Soyka and Heyer 1999, Pérez-Amador et al. 2002, Urano et al. 2004). Putrescine is also required for the synthesis of tropane and nicotine alkaloids. The first key step of the biosynthesis of these alkaloids is catalyzed by putrescine N-methyltransferase (PMT). Because the sequences of PMT and SPDS are similar to each other, the gene for PMT may have been derived from the gene for SPDS (Hibi et al. 1994, Biastoff et al. 2009). The Arabidopsis genome contains two genes encoding SPDS, SPDS1 and SPDS2. The spds1 spds2 double mutant shows embryonic lethality, indicating the essential role of spermidine (Imai et al. 2004a). It is possible that some roles of spermidine can be replaced by spermine. The absolute requirement for spermidine for survival has been shown in yeasts, and at least one of the reasons is because of the specificity of spermidine as a necessary substrate for the hypusine modification of the eukaryotic initiation factor 5A (eIF5A; Park et al. 2010). The aminobutyl moiety of spermidine is transferred to a specific lysine in the eIF5A precursor by deoxyhypusine synthase (DHS). The deoxyhypusine residue is then hydroxylated to form hypusine. Hypusinated eIF5A is essential for growth and cell viability in yeasts. It is likely that this is also the case in plants and animals. In some plant species, the aminobutyl moiety of spermidine is also transferred to putrescine by homospermidine synthase (HSS) to form homospermidine, which is an essential precursor in the synthesis of pyrrolizidine alkaloids, defense compounds against herbivores (Ober and Kaltenegger 2009). The tetramine spermine is known to regulate a number of ion channels and receptors, in particular the inward rectifying potassium channels in mammalian cells (Williams 1997). In higher plants, a number of studies have shown that spermine functions as a signaling molecule in disease resistance mechanism (Kusano et al. 2008, Alcázar et al. 2010). Because degradation of spermine by polyamine oxidase (PAO) results in the production of H$_2$O$_2$, this oxidative degradation may trigger defense responses to biotic or abiotic stresses (Walters 2003, Cona et al. 2006). The spms-1 mutant of Arabidopsis is defective in the synthesis of spermine and has been found to be more sensitive to high salt and drought stresses than the wild type (Yamaguchi et al. 2007). However, this mutant shows no morphological aberrations under normal growth conditions (Imai et al. 2004b).
Therefore, unlike mammals in which spermine deficiency results in an X-linked mental retardation disorder known as the Snyder–Robinson syndrome and in deafness (Wang et al. 2009, Schwartz et al. 2011), higher plants do not always need spermine for growth. Spermidine is also converted to thermspermine, a structural isomer of spermine, by thermspermine synthase (TSPMS). In contrast to spermine, thermspermine has been shown to be required for normal growth and development by studies of thermspermine-deficient aculis5 (acl5) mutants of Arabidopsis (Kakehi et al. 2008). Furthermore, recent genome analyses in many organisms suggest widespread distribution of thermspermine in the plant kingdom (Fuell et al. 2010, Pegg and Michael 2010). In this review, we focus on thermspermine in terms of its homeostasis, physiological function, and the mode of action with reference to relevant information on other polyamines. More comprehensive reviews on plant polyamines are available elsewhere (Kusano et al. 2008, Alca´zar et al. 2010, Takahashi and Kakehi 2010).

Fig. 2 Phenotype of thermspermine-defective acl5 mutants. (A) Gross morphology of wild-type (left) and acl5 (right) adult flowering plants. (B) Cross-section of the wild-type stem stained with phloroglucinol. (C) Cross-section of the acl5 mutant stem stained with phloroglucinol.

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Origin of thermspermine synthase
Thermspermine was first identified in the therophilic bacterium, Thermus thermophilus (Oshima 1979). However, it was not until 2007 that the gene for TSPMS was identified in the diatom Thalassiosira pseudonana and in Arabidopsis (Knott et al. 2007). Meanwhile, the Arabidopsis ACLS gene, which is now assigned as the gene for TSPMS, was cloned from the acl5 mutant but misidentified to code for SPMS (Hanzawa et al. 2000) because thermspermine is indistinguishable from spermine in the standard analysis of polyamines by dansylation followed by HPLC. These isomers can be separated by their benzoylation in the HPLC analysis (Fig. 3; Naka et al. 2010).

The acl5 mutant shows a severe dwarfism (Fig. 2), and the phenotype is partially rescued by exogenous treatment with thermspermine but not with spermine, indicating an absolute requirement for thermspermine for stem elongation in Arabidopsis (Kakehi et al. 2008). According to database searches, ACLS gene homologs are widespread in the plant kingdom including the above-mentioned diatoms, water molds (oomycetes), brown algae, green algae, mosses, liverworts, ferns and gymnosperms, but are absent in animals and fungi, while genes for SPMS occur in animals, fungi and angiosperms, but have not been identified so far in lower plants such as algae, mosses and ferns (Fig. 4). A study on the origin of polyamine biosynthetic genes suggests that, while SPMS may have independently arisen from SPDS at least three times during the evolution of eukaryotes, i.e. animals, fungi and higher plants, TSPMS has been acquired by an ancestor of the plant lineage through horizontal gene transfer from archaea or bacteria (Minguet et al. 2008). Thermus thermophilus has only one ACLS gene homolog, SpeE, and this gene is assigned to encode bifunctional triamine/agmatine aminopropyltransferase (TAAPT; Ohnuma et al. 2011). Both bacterial and archaeal thermophiles have recently been shown to possess a unique pathway to synthesize spermidine in which agmatine is converted by TAAPT to N1-propylagmatine which is then hydrolyzed to form spermidine (Ohnuma et al. 2005, Morimoto et al. 2010). An ACLS-like TAAPT gene is also present in cyanobacteria. Although TSPMS activity has not been detected in
Regulation of thermospermine biosynthesis

The amount of thermospermine in whole-cell extracts from Arabidopsis seedlings is several fold lower than that of spermine (Fig. 3; Naka et al. 2010). This may be partly due to the difference in expression patterns of ACL5 and SPMS genes. While SPMS mRNA is expressed ubiquitously, ACL5 mRNA is limited to procambial cells and xylem precursor cells during vascular differentiation (Clay and Nelson 2005, Muñiz et al. 2008). Furthermore, unlike ACL5, SPMS tightly interacts with SPD51 and SPD52, and forms a complex named a polyamine metabolon, suggesting that spermine is produced more efficiently by this complex (Panicot et al. 2002). The level of the acl5-1 missense mRNA in the acl5-1 allele is much higher than that of the ACL5 mRNA in the wild type, and both acl5-1 and ACL5 mRNA levels are decreased by exogenous thermospermine in the respective plants, indicating that ACL5 expression is under negative feedback control by thermospermine (Kakehi et al. 2008). In contrast, expression of SPD51, SPD52 and SPMS in Arabidopsis is not responsive to exogenous polyamines, indicating no direct involvement of transcriptional regulation of these genes in cellular homeostasis of spermidine and spermine (Kakehi et al. 2008). SPMS expression is increased in response to ABA (Hanzawa et al. 2002), and this is consistent with proposed roles of spermine in stress responses such as high salt and drought. As described later, ACL5 expression is enhanced by auxin (Hanzawa et al. 2000).

Intracellular levels of polyamines may be regulated by multiple mechanisms involving biosynthesis, conjugation, degradation and transport. As mentioned in the Introduction, ODC catalyzes an initial and rate-limiting step in polyamine biosynthesis in animals and fungi and is negatively regulated by high levels of polyamines through the interaction with ODC antizyme. Antizyme was originally identified as a protein inhibitory to ODC and targets ODC for proteasomal degradation (Murakami et al. 1992). Genes encoding ODC antizyme contain two partially overlapping open reading frames (ORFs) and, when intracellular polyamine levels are high, the full-length active protein is synthesized by a conserved +1 ribosomal frameshifting mechanism that enables bypass of the internal stop codon (Matsufuji et al. 1995, Ivanov et al. 2000). A recent study has shown that, while the frameshifting just causes translational pausing and reduces the rate of translation, polyamine binding to nascent antizyme polypeptide promotes completion of its synthesis (Kurian et al. 2011). Unlike in other eukaryotes, however, ODC antizyme has not been identified in plants. It remains unknown whether this polyamine-dependent translational control can be functional in plant cells or not.

On the other hand, as shown in early studies of the effect of methylglyoxal bis(guanylylhydrazone) (MGBG), a polyamine analog on tobacco cells (Hiatt et al. 1986), AdoMetDC plays a key regulatory role in providing an aminopropyl moiety for the synthesis of higher polyamines. In mammals, AdoMetDC mRNA has a S′ leader containing a short upstream open reading frame (uORF) that codes for the hexapeptide MAGDIS.
Elevated polyamines inhibit synthesis of this peptide by stabilizing a ribosome paused in the vicinity of its termination codon and consequently block AdoMetDC translation (Hill and Morris 1993, Ruan et al. 1996). The Arabidopsis AdoMetDC1 mRNA contains two overlapping uORFs. At low polyamine concentrations, the first and tiny uORF has an inhibitory effect on translation of the second overlapping uORF and instead acts in facilitating translation of the AdoMetDC-coding frame. Elevated polyamines bypass the effect of the first uORF and lead to translation of the second uORF, which prevents the AdoMetDC-coding frame from being translated (Hanfrey et al. 2005). A similar uORF-mediated translational control of AdoMetDC expression may be widespread because uORFs are found in the 5′ leader sequences of AdoMetDC in diverse organisms (Ivanov et al. 2010). Interestingly, among the four genes for AdoMetDC in Arabidopsis, only AdoMetDC4/BUD2 has no uORF and its expression is negatively regulated by exogenous thermospermine in a manner similar to ACL5 expression (Kakehi et al. 2010). Given that loss-of-function mutants of AdoMetDC4/BUD2 show bushy and dwarf phenotypes that are not identical but very similar to acl5 phenotype (Ge et al. 2006), it is likely that AdoMetDC4/BUD2 is preferentially associated with the synthesis of thermospermine.

In the biosynthesis of spermidine, spermine and thermospermine, the 3-aminopropyl donor dcAdoMet is converted to 5′-methylthioadenosine (MTA; Fig. 1). Thus, it is possible that cellular MTA levels affect polyamine biosynthesis. In plants, MTA is hydrolyzed to 5-methylthioribose and adenine by MTA nucleosidase (MTN) and is subsequently recycled to methionine via the methionine cycle (Miyazaki and Yang 1987). Knock-down mutants of MTN genes in Arabidopsis display delayed flowering and have non-fertile flowers and, when grown with MTA as a sulfur source, they have elevated levels of putrescine and spermine (Bürstenbinder et al. 2010). The effect of altered MTA metabolism on thermospermine synthesis is not yet fully understood, but it is clear that MTA plays a key role in regulating polyamine metabolism.
biosynthesis, and vice versa, remains to be addressed in future experiments.

**Transport, conjugation and catabolism**

Although de novo synthesis may be the main source of polyamines in cells, their uptake and transport can contribute to homeostasis of polyamines. However, little information is available on uptake and transport mechanisms for polyamines in plants. In bacteria and yeasts, multiple transporters for polyamines have been identified (Kashiwagi and Igarashi 2011). In yeast plasma membranes these include a polyamine/amino acid permease, AGP2, a polyamine/urea transporter, DUR3, a polyamine/AdoMet/amino acid transporter, SAM3, and five efflux pumps for polyamines, TPO1–TPO5. UGA4 functions as a polyamine/AdoMet/amino acid transporter, SAM3, and five acid permease, AGP2, a polyamine/urea transporter, DUR3, a yeast plasma membranes these include a polyamine/amino

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promotes xylem development, excess proliferation of xylem vessels in the radial direction in the mutant stem might reflect changes in auxin flow from the axial to the radial direction. The dwarf phenotype with rather thin stems of acl5/tkv mutants appears to be attributed to reduction in a cell population that normally differentiates into parenchyma and leads to stem growth. Another study suggests that ACL5 prevents premature cell death of developing xylem vessels and functions in the correct specification of xylem cells, based on the observation that the hypocotyl of the acl5 mutant has predominantly protoxylem vessels with spiral cell wall patterning, while pitted-type metaxylem vessels and xylem fibers are completely missing (Muñiz et al. 2008). Xylem fibers and the elaborate pitted type of vessel elements are also absent in acl5 stems (Vera-Sirera et al. 2010). In the process of xylem development, uncommitted cells differentiate into procambium precursor cells, procambial cells and then xylem precursor cells, from which xylem vessels, xylem fibers and xylem parenchyma cells are derived (Ohashi-Ito and Fukuda 2010). Detailed expression analyses reveal that ACL5 expression is confined to xylem vessel elements as well as procambial cells and, in particular, to metaxylem vessels in the root tissue (Muñiz et al. 2008). Is ACL5 then required for differentiation of metaxylem vessels and xylem fibers? It seems contradictory that acl5 mutants show increased expression of VND7 and VND6 (Muñiz et al. 2008), which encode NAC-domain transcription factors and play a key role in the differentiation of protoxylem and metaxylem, respectively (Kubo et al. 2005). We have confirmed that expression of SND1, which encodes another NAC-domain transcription factor involved in secondary wall synthesis in fibers (Zhong et al. 2006), is not reduced in acl5 (unpublished). Furthermore, expression of ATHB8, a member of the class III homeodomain-leucine zipper (HD-Zip III) protein gene family, is increased in acl5 and down-regulated by exogenous thermospermine (Kakehi et al. 2008). Conversely, knock-down of the HD-Zip III genes by transgenic overexpression of microRNA miR165, which targets all the five HD-Zip III genes, results in the reduced expression of ACL5 and a subset of the genes related to vascular development (Zhou et al. 2007). The HD-Zip III genes drive the de novo xylem formation (Carlsbecker et al. 2010, Ilegems et al. 2010). In particular, ATHB8 is expressed under the control of a central regulator of auxin signaling, MONOPTEROS (MP)/AUXIN RESPONSE FACTORS (ARFs), in procambium precursor and procambial cells (Donner et al. 2009). Taken together with the fact that ACL5 expression is itself responsive to auxin, it is suggested that thermospermine functions in the repression of auxin-dependent xylem development rather than in the specification of xylem cell types. MP directly expression of PINFORMED1 (PIN1) encoding an auxin efflux carrier and integrates a positive feedback loop of auxin flow with PIN1, which is consistent with the canalization-of-auxin-flow hypothesis (Wenzel et al. 2007). This feedback loop has to be kept confined along the direction of veins to be formed. Thermospermine appears to act as an anti-auxin in limiting this feedback.

### Molecular mode of action of thermospermine

In an effort to elucidate the precise mode of action of thermospermine, suppressor mutants of acl5, named sac, that restore the stem growth in the absence of thermospermine have been isolated. The sac51-d and sac52-d mutations show dominant inheritance and their responsible genes encode a basic helix–loop–helix (bHLH) transcription factor and a ribosomal protein L10 (RPL10) (RPL10), respectively (Imai et al. 2006, Imai et al. 2008). The SAC51 mRNA contains five uORFs within the 0.5 kb long 5' leader sequence and the sac51-d allele has a single base substitution in the fourth uORF that causes a premature stop codon with a large truncation of its deduced polypeptide. Since this uORF has an inhibitory effect on the translation of the main ORF in acl5, thermospermine may act in bypassing this effect (Imai et al. 2006). In sac51-d acl5-1 double mutants, disruption of the fourth uORF appears to suppress the deficiency in thermospermine and consequently leads to translation of the main ORF (Fig. 5). This scenario is consistent with the dominant nature of the sac51-d phenotype, if the bHLH transcription factor encoded by the SAC51 main ORF is required for promotion of stem elongation, namely in this case repression of xylem differentiation. Furthermore, sac52-d may also evade the effect of the fourth uORF of SAC51 (Imai et al. 2008). RPL10 is a key protein in assembling the 60S ribosomal subunit and organizing the aminoaicyl-tRNA binding site for mRNA translation. The sac52-d allele might provide a dominant positive form of RPL10 that helps a possibly stalled ribosome to be released from the SAC51 fourth ORF or alternatively promotes leaky scanning of a ribosome through uORFs. Taking into account that most intracellular polyamines exist in a polyamine–RNA complex (Igarashi and Kashwagi 2010), it is possible that thermospermine stabilizes the secondary structure of the S' leader region of the SAC51 mRNA that allows the scanning ribosome to reach the main ORF efficiently (Fig. 5). Polyamine-dependent stabilization of the bulged out region of...
double-stranded RNA in mRNA has been shown in bacterial and mammalian cells (Igarashi and Kashwagi 2011). Alternatively, thermospermine might interact with rRNA, ribosomal proteins (e.g. RPL10) and/or nascent polypeptides translated from the fourth uORF of SACS1 to release the stalled ribosome. The nascent polypeptide chain that causes ribosome stalling and regulates translation is found in most cases to be encoded as uORFs or as N-terminal leader peptides (Morris and Geballe 2000, Tenson and Ehrenberg 2002), as mentioned above on the mammalian MAGDIS uORF. Nascent peptide-dependent translational arrest at the N-terminal coding sequence has been found to occur in response to AdoMet in the Arabidopsis CGS1 gene, which encodes cystathionine γ-synthase, a key enzyme of methionine biosynthesis in plants (Onoue et al. 2011).

Exogenous application of spermine cannot rescue the stem growth of acl5 (Hanzawa et al. 2000, Kakehi et al. 2008). We have found that norspermine can substitute for thermospermine in rescuing the mutant phenotype (Kakehi et al. 2010). It is noted that the NC3NC4N arrangement of carbon chains is present in both thermospermine (NC3NC4NC6N) and norspermine (NC3NC4NC4N), but not in spermine (NC4NC4NC6N). More detailed biochemical studies on this core structure will help to elucidate the precise mode of action of thermospermine. Furthermore, studies on additional sac mutants are necessary for full understanding of the function of thermospermine.

**Future perspectives**

Is thermospermine a phytohormone? According to a strict definition of phytohormones, they are supposed to function in plant growth and development at a site remote from their place of production. Although exogenous thermospermine is indeed bioactive, coincidence of the tissues expressing ACL5 with those manifesting the abnormality in acl5 mutants suggests that thermospermine functions autonomously in the cells where it is synthesized. In contrast to well-known phytohormones, which are recognized by specific receptors and play a variety of roles in many aspects of plant growth, thermospermine may directly target the translation machinery for specific genes. We thus conclude that thermospermine is a novel type of plant growth regulator that has a prokaryotic origin. However, the possibility should not be excluded that thermospermine has another mode of action, given its versatility as a small polycation. While its function may have become specialized for negative control of xylem development during the evolution of vascular plants, its widespread distribution in the plant kingdom suggests the functional significance of thermospermine as a fundamental molecule. One of the most important questions to be answered is: what is the function of thermospermine in non-vascular plants? As for vascular plants, it should be examined whether or not SACS1 or its ortholog is a principal target of thermospermine in the control of xylem development. So far there is no direct evidence showing the function of SACS1 as a repressor of xylem development. Finally, the full understanding of the function of thermospermine in xylem development will surely be important for woody biomass production from a biotechnological point of view.

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