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Review

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 *ACAULIS5* (*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, ACAULIS5; ADC, arginine decarboxylase; AdoMetDC/SAMDC, *S*-adenosylmethionine decarboxylase; bHLH, basic helix–loop–helix; BUD2, BUSHY AND DWARF2; dcAdoMet/dcSAM, decarboxylated *S*-adenosylmethionine; eIF5A, eukaryotic initiation factor 5A; GABA, γ-aminobutyric acid; MP, MONOPTEROS; MTA, 5′-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/agmatine 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

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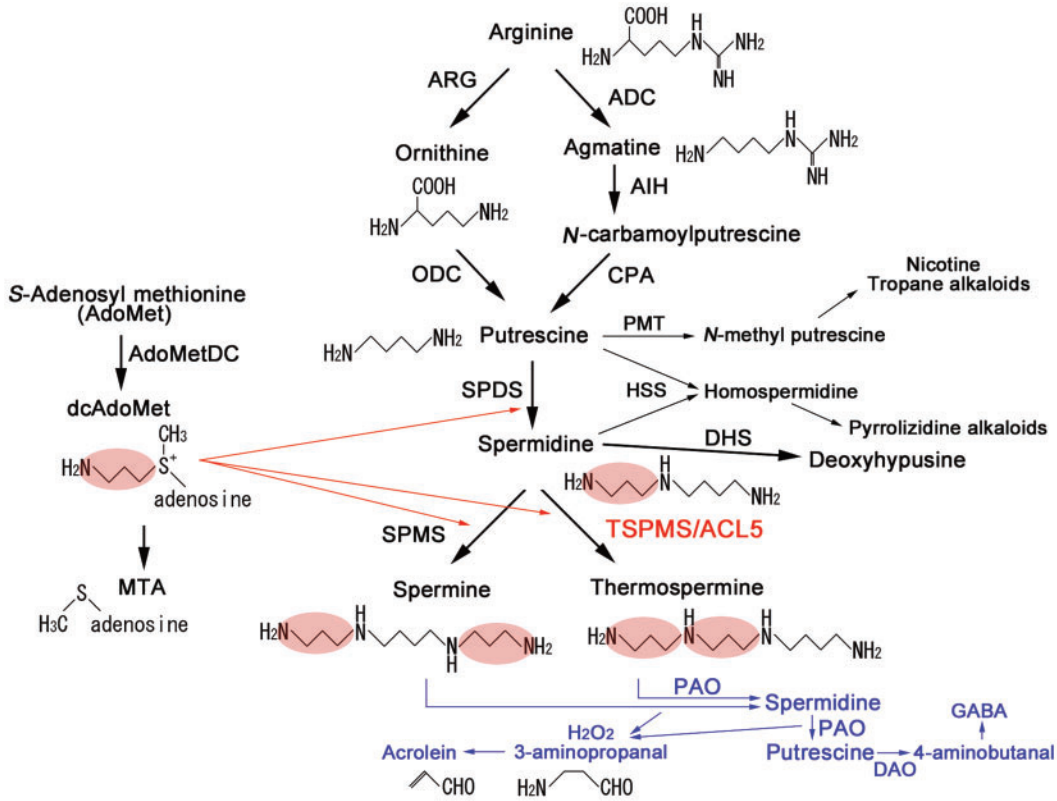

Fig. 1 Polyamine biosynthesis and catabolism networks in plants. ARG, arginase; ODC, ornithine decarboxylase; ADC, arginine decarboxylase, AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; SPDS, spermidine synthase; PMT, putrescine *N*-methyltransferase; HSS, homospermidine synthase; DHS, deoxyhypusine synthase; SPMS, spermine synthase; TSPMS, thermospermine synthase; AdoMetDC, *S*-adenosylmethionine decarboxylase; MTA, methylthioadenosine; PAO, polyamine oxidase; DAO, diamine oxidase; GABA, γ-aminobutyric acid.

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_2O_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).

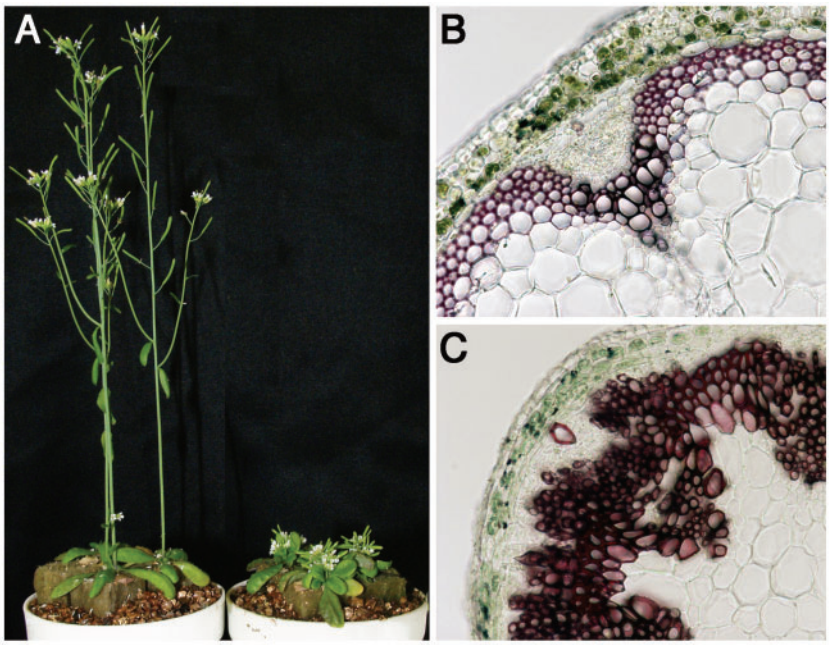

Fig. 2 Phenotype of thermospermine-defective *acl5* mutants. (A) Gross morphology of wild-type (left) and *acl5* (right) adult flowering plants. (B) Cross-section of the wild-type stem stained with phloroglucinol. (C) Cross-section of the *acl5* mutant stem stained with phloroglucinol.

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 thermospermine, a structural isomer of spermine, by thermospermine synthase (TSPMS). In contrast to spermine, thermospermine has been shown to be required for normal growth and development by studies of thermospermine-deficient *acaulis5* (*acl5*) mutants of Arabidopsis (**Fig. 2**; Kakehi et al. 2008). Furthermore, recent genome analyses in many organisms suggest widespread distribution of thermospermine in the plant kingdom (Fuell et al. 2010, Pegg and Michael 2010). In this review, we focus on thermospermine 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, Alcázar et al. 2010, Takahashi and Kakehi 2010).

Origin of thermospermine synthase

Thermospermine was first identified in the thermophilic 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 *ACL5* 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 thermospermine 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 thermospermine but not with spermine, indicating an absolute requirement for thermospermine for stem elongation in Arabidopsis (Kakehi et al. 2008). According to database searches, *ACL5* 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 *ACL5* 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 N^1-propylagmatine which is then hydrolyzed to form spermidine (Ohnuma et al. 2005, Morimoto et al. 2010). An *ACL5*-like TAAPT gene is also present in cyanobacteria. Although TSPMS activity has not been detected in

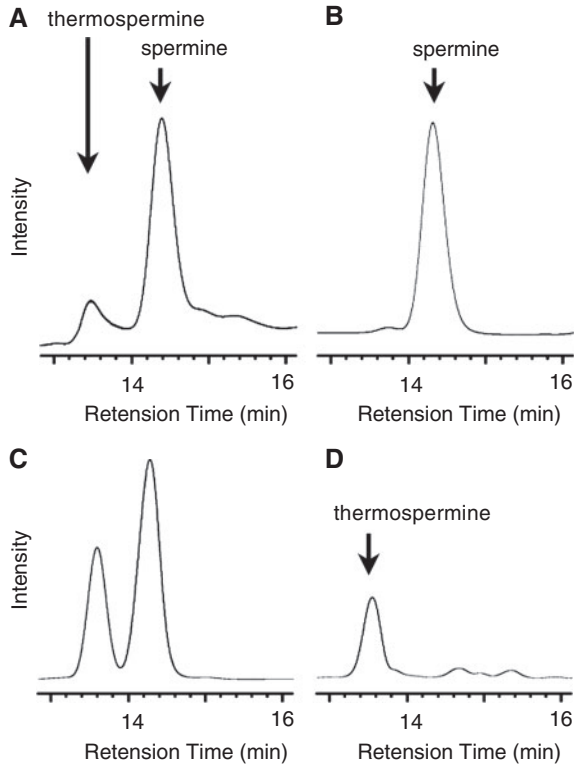

Fig. 3 Detection of thermospermine and spermine in plant extracts by HPLC. (A) HPLC chromatogram of the cell extract from Arabidopsis wild-type inflorescences. (B) HPLC chromatogram of the cell extract from Arabidopsis *acl5* inflorescences. (C) HPLC chromatogram of the cell extract from young ginkgo nuts. (D) HPLC chromatogram of the cell extract from leafy gametophytes of *Physcomitrella patens*.

these TAAPTs or as yet fully examined, the high degree of similarity between *ACL5* and prokaryotic TAAPT genes suggests that plant TSPMS has originated from prokaryotic TAAPT. Acquisition of TSPMS through the above-mentioned gene transfer might have occurred from a cyanobacterial ancestor of the chloroplast to the host algal nucleus (Fuell et al. 2010). We have confirmed by HPLC analysis that thermospermine was detected in extracts from *Ginkgo biloba* (gymnosperm), *Chlamydomonas reinhardtii* (green alga) and *Phytophthora infestans* (oomycete), while spermine was undetectable in those from *Chlamydomonas* and *Phytophthora* (unpublished). Our data also revealed that ginkgo nuts contained both thermospermine and spermine (**Fig. 3**), although the gene for SPMS has not yet been identified in gymnosperms. Transgenic expression of an *ACL5* homolog of *Physcomitrella patens* in Arabidopsis *acl5* mutants can significantly complement the dwarf phenotype (Takahashi and Kakehi 2010). Taken together with the widespread occurrence of *ACL5* gene homologs, we conclude that thermospermine is not just a minor polyamine in the plant kingdom. In contrast, the absence of flowering plant-type genes for SPMS in algae, mosses and ferns might indicate a limited distribution of spermine in the plant kingdom, although it is possible that, due to broad substrate specificity, SPDS homologs possess SPMS activity in some cases.

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 SPDS1 and SPDS2, 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 *SPDS1*, *SPDS2* 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(guanylhydrazone) (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 5′ leader containing a short upstream open reading frame (uORF) that codes for the hexapeptide MAGDIS.

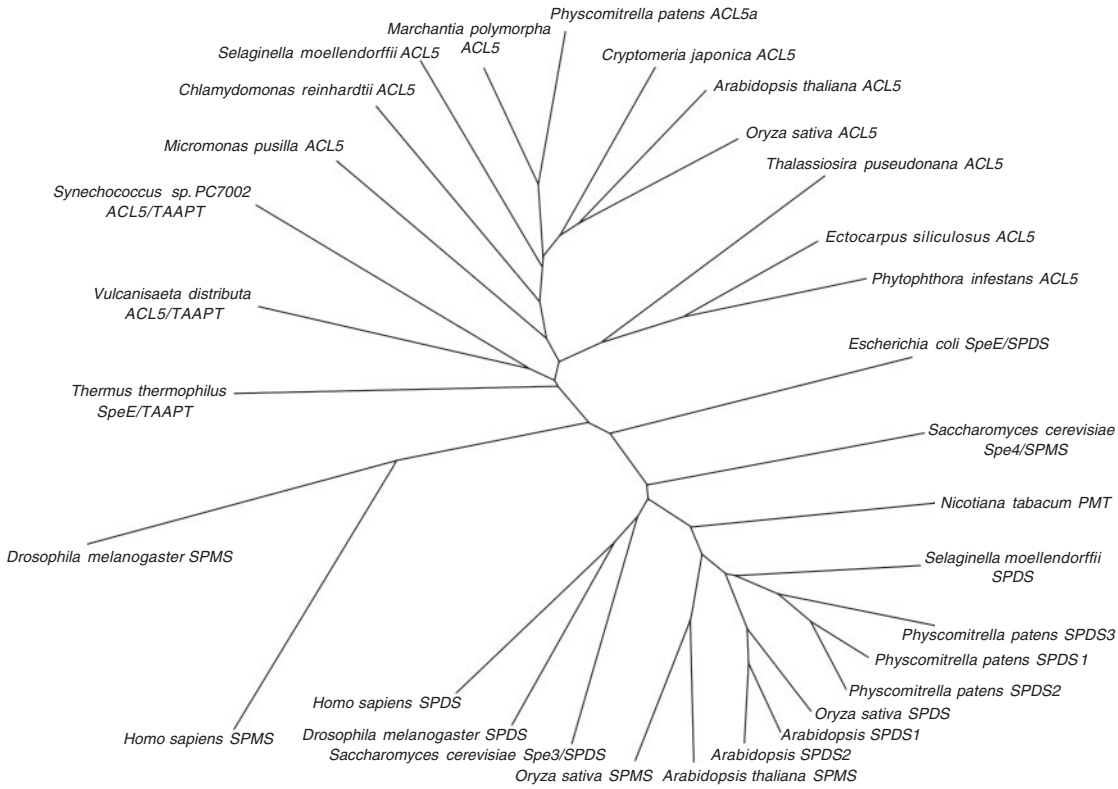

Fig. 4 Phylogenetic relationship of the polyamine aminopropyltransferase proteins. The unrooted tree was constructed using Neighbor–Joining methods after making a multiple alignment of amino acid sequences with the Clustal X program. The proteins that exhibit sequence similarity to Arabidopsis ACL5/TSPMS were retrieved by using PSI-BLAST, except for those of liverworts which were provided by Dr. T. Kohchi. Accession numbers are Arabidopsis *ACL5* (NP_568376), Arabidopsis *SPDS1* (NP_173794), Arabidopsis *SPDS2* (NP_177188), Arabidopsis *SPMS* (NP_568785), *Chlamydomonas ACL5* (ADF43120), *Cryptomeria ACL5* (BAC82351), *Drosophila SPDS* (NP_731384), *Drosophila SPMS* (NP_729798), *Ectocarpus ACL5* (CBJ31336), *Escherichia SpeE/SPDS* (NP_414663), *Homo SPDS* (NP_003123), *Homo SPMS* (NP_004586), *Micromonas ACL5* (XP_002500355), *Nicotiana PMT* (AAF14879), *Oryza ACL5* (NP_001046395), *Oryza SPDS* (NP_001059438), *Oryza SPMS* (NP_001057773), *Physcomitrella ACL5a* (XP_001762338), *Physcomitrella SPDS1* (XP_001752964), *Physcomitrella SPDS2* (XP_001757204), *Phytophthora ACL5* (XP_002896487), *Saccharomyces Spe3/SPDS* (NP_015394), *Saccharomyces Spe4/SPMS* (NP_013247), *Selaginella ACL5* (XP_002986373), *Selaginella SPDS* (XP_002974059), *Synechococcus ACL5/TAAPT* (YP_001735517), *Thalassiosira ACL5* (TPS_41289), *Thermus SpeE/TAAPT* (YP_004447) and *Vulcanisaeta ACL5/TAAPT* (YP_003900948).

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 the *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

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 γ-aminobutyric acid (GABA)/putrescine peamease in the yeast vacuolar membrane. Putative orthologs of some of these proteins are found in plants, suggesting the possibility that multiple types of transporters may participate in the transport of polyamines. Based on sequence data and results from complementation experiments of yeast *agp2* mutants, a gene named *Polyamine Uptake Transporter 1* (*PUT1*) was recently identified in rice and proposed to encode a spermidine-preferential transporter (Mulangi et al. 2012). Long-distant transport of polyamines in higher plants has been shown to occur via xylems and phloems (Friedman et al. 1986, Antognoni et al. 1998).

In addition to free polyamines, conjugates of polyamines with hydroxycinnamic acids, which are referred to as hydroxycinnamic acid amides (HCAAs), are detected in plant cells (Martin-Tanguy 1985, Bagni and Tassoni 2001). These conjugates have been implicated in defense responses against pathogens, detoxification of phenolic compounds, and also as a reservoir of polyamines. The genes for spermidine disinapoyltransferase (SDT), spermidine dicoumaroyltransferase (SCT) and spermidine hydroxycinnamoyltransferase (SHT) have been cloned from Arabidopsis, but the mutants of the respective genes show normal growth (Grienenberger et al. 2009, Luo et al. 2009). Polyamines are also covalently bound to glutamine residues in certain proteins by transglutaminase (TGase; Serafini-Fracassini and Del Duca 2008). For instance, a cross-link of polyamines with cytoskeleton proteins such as tubulin and actin has been found in the pollen tubes of some plants (Del Duca et al. 1997). The Arabidopsis *PNG1* gene encoding a peptide:*N*-glycanase was previously identified as a gene for TGase but was later shown to encode a bona fide glycanase (Diepold et al. 2007). Thus, further studies are needed to identify genes encoding TGase. There is so far no information on the conjugation of thermospermine.

Thermospermine and spermine can also be substrates for long-chain polyamines. In diatoms, long-chain polyamines are required for silica precipitation during cell wall formation. Close inspection of diatom genomes reveals that they have gene fusions of AdoMetDC and an aminopropyltransferase that are probably derived from bacteria, and these gene products may potentially act in the iterative addition of multiple aminopropyl groups to form long-chain polyamines (Michael 2011). There are, however, no orthologs of these fusion genes in other Stramenopiles and land plants.

As regards degradation, polyamines are oxidized by FAD-dependent PAOs. There exist two types of the enzymes in plants. Apoplastic PAOs that were characterized in maize and barley oxidize spermine and spermidine to produce *N*-(3-aminopropyl)-4-aminobutanal and 4-aminobutanal, respectively, along with 1,3-diaminopropane and H_2O_2 (Cona et al. 2006). The PAOs localized in peroxisomes or the cytosol back-convert spermine and spermidine to spermidine and putrescine, respectively, along with 3-aminopropanal and H_2O_2 in a manner similar to animal PAOs. Arabidopsis has five PAO isoforms, among which PAO1, PAO2, PAO3 and PAO4 have been shown to act in a polyamine back-conversion pathway (Tavladoraki et al. 2006, Moschou et al. 2008, Kamada-Nobusada et al. 2008, Takahashi et al. 2010, Fincato et al. 2011). In particular, PAO1 with a predicted cytosolic localization has been shown to oxidize thermospermine preferentially in vitro (Takahashi et al. 2010, Fincato et al. 2011). Although PAO5 remains to be characterized, a molecular phylogenetic tree of the plant PAO family indicates that the Arabidopsis genome has no gene for apoplastic PAO, while the rice genome has three genes belonging to the class that contains maize and barley apoplastic PAOs (Ono et al. 2012). Most studies on the function of spermine in biotic and abiotic stress responses in plants have emphasized the importance of H_2O_2 derived from spermine oxidation since it mediates cell death, hypersensitive responses and expression of defense genes. However, it should also be noted that 3-aminopropanal generated by polyamine back-conversion is a highly reactive aldehyde and is spontaneously deaminated to give acrolein (**Fig. 1**). In mammalian cell cultures, the toxicity of acrolein is higher than that of H_2O_2 (Yoshida et al. 2009). Exogenous application of high concentrations of polyamines is toxic to the growth of plants (Kakehi et al. 2008). This might be attributed primarily to the production of acrolein. The effect of polyamine-derived acrolein on growth and stress responses in plants should be investigated in depth in future studies.

Functions of thermospermine in plant growth

Initial characterization of the *acl5* mutant of Arabidopsis revealed that the dwarf phenotype of the mutant is associated with overproliferation of xylem vessels (**Fig. 2**; Hanzawa et al. 1997). In addition, the *thickvein* (*tkv*) mutant displaying thicker veins in leaves and stems together with dwarfism was found to be another loss-of-function allele of *ACL5* (Clay and Nelson 2005). Since the *tkv* mutant shows a significant reduction in polar auxin transport along the inflorescence stem, the defect in auxin transport is suggested to be responsible for the vascular phenotypes (Clay and Nelson 2005). Considering that auxin

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 FACTOR5* (*ARF5*), 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* directs 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), 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 fourtn uORF of *SAC51* (Imai et al. 2008). RPL10 is a key protein in assembling the 60S ribosomal subunit and organizing the aminoacyl-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 uORF 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 Kashiwagi 2010), it is possible that thermospermine stabilizes the secondary structure of the 5′ 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

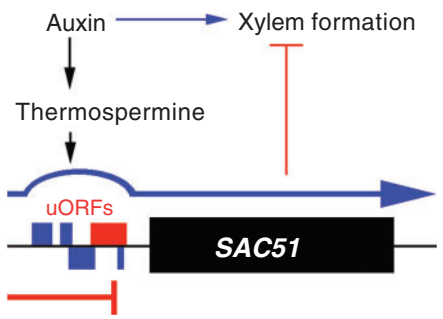

Fig. 5 Hypothetical model for the molecular mode of action of thermospermine in auxin-induced xylem formation. The black bar represents *SAC51* mRNA. The main ORF encoding a bHLH transcription factor, the fourth uORF, which has been shown to be inhibitory to translation of the main ORF, and another four uORFs are indicated by black, red and blue boxes, respectively. Thermospermine appears to play a role opposite to that of auxin in xylem formation, probably by enhancing the translation of *SAC51*.

double-stranded RNA in mRNA has been shown in bacterial and mammalian cells (Igarashi and Kashiwagi 2011). Alternatively, thermospermine might interact with rRNA, ribosomal proteins (e.g. RPL10) and/or nascent polypeptides translated from the fourth uORF of *SAC51* 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 NC_3NC_3N arrangement of carbon chains is present in both thermospermine ($NC_3NC_3NC_4N$) and norspermine ($NC_3NC_3NC_3N$), but not in spermine ($NC_3NC_4NC_3N$). 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 *SAC51* 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 *SAC51* 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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