The *SAC51* family plays a central role in thermospermine responses in *Arabidopsis*

Running title: The *SAC51* family in *Arabidopsis*

Qingqing Cai[1], Hiroko Fukushima[1], Mai Yamamoto[1], Nami Ishii[1], Tomoaki Sakamoto[2], Tetsuya Kurata[2, §], Hiroyasu Motose[1] and Taku Takahashi[1]

[1]Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan

[2]Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Japan

Abbreviations: ACL5, ACAULIS5; ATHB8, ARABIDOPSIS THALIANA HOMEOBOX8; bHLH, basic helix-loop-helix; 2,4-D IOE, *isooctyl ester* of 2,4-dichlorophenoxyacetic acid; EMS, ethyl methanesulfonate; GUS, *β-glucuronidase;* LHW, LONESOME HIGHWAY; mORF, main open-reading frame; MP, MONOPTEROS; PAO, POLYAMINE OXIDASE; SAC, SUPPRESSOR-OF-ACL5; SACL, SAC-LIKE; SAMDC, S-adenosylmethionine decarboxylase; TMO5, TARGET OF MP5; T5L1, TMO5-LIKE1; uORF, upstream open-reading frame

[§]Present address: Graduate School of Life Sciences, Tohoku University, Sendai, Japan.

Abstract

The *acaulis5* (*acl5*) mutant of *Arabidopsis thaliana* is defective in the biosynthesis of thermospermine and shows a dwarf phenotype associated with excess xylem differentiation. *SAC51* was identified from a dominant suppressor of *acl5*, *sac51-d*, and encodes a basic helix-loop-helix protein. The *sac51-d* mutant has a premature termination codon in an upstream open-reading frame (uORF) that is conserved among all four members of the *SAC51* family, *SAC51* and *SACL1* to *SACL3*. This suggests that thermospermine cancels the inhibitory effect of the uORF in main ORF translation. Another suppressor, *sac57-d*, has a mutation in the conserved uORF of *SACL3*. To define further the function of the *SAC51* family in the thermospermine response, we analyzed T-DNA insertion mutants of each gene. Although *sacl1-1* may not be a null allele, the quadruple mutant showed a semi-dwarf phenotype but with an increased level of thermospermine and decreased sensitivity to exogenous thermospermine that normally represses xylem differentiation. The *sac51-1 sacl3-1* double mutant was also insensitive to thermospermine. These results suggest that *SAC51* and *SACL3* play a key role in thermospermine-dependent negative control of thermospermine biosynthesis and xylem differentiation. Using 5' leader-*GUS* fusion constructs, however, we detected a significant enhancement of the GUS activity by thermospermine only in *SAC51* and *SACL1* constructs. Furthermore, while *acl5-1 sac51-1* showed the *acl5* dwarf phenotype, *acl5-1 sacl3-1* exhibited an extremely tiny-plant phenotype. These results suggest a complex regulatory network for the thermospermine response in which *SAC51* and *SACL3* function in parallel pathways.

Keywords: Arabidopsis, thermospermine, translation, uORF, xylem differentiation

Introduction

Polyamines are small aliphatic amines found in all living organisms and are involved in a variety of biological processes such as mRNA translation, protein modification, and ion channel modulation (Tabor and Tabor 1984, Cohen 1998, Igarashi and Kashiwagi 2010, Pegg and Casero 2011). Thermospermine, a tetraamine structural isomer of spermine, is present in some bacteria and ubiquitously in plants (Minguet et al. 2008, Takano et al. 2012). In *Arabidopsis thaliana*, *ACAULIS5* (*ACL5*) encodes thermospermine synthase (Knott et al. 2007, Kakehi et al. 2008). Its loss-of-function mutant, *acl5*, shows a dwarf phenotype with over-proliferation of xylem vessels (Hanzawa et al. 1997), but also lacks in xylem fibers (Muñiz et al. 2008, Vera-Sirera et al. 2010). Analysis of *thickvein* (*tkv*), another mutant *ACL5* allele, suggests that *ACL5* is involved in polar auxin transport for proper vein development (Clay and Nelson 2005). When exogenously supplied, thermospermine and norspermine, which both contain two tandemly arrayed trimethylene groups, partially restore the growth of *acl5*, and thus, appear to act as plant growth regulators (Kakehi et al. 2010). *ACL5* expression is restricted to differentiating xylem vessels (Muñiz et al. 2008), upregulated by both auxin and the *acl5* mutation (Hanzawa et al. 2000), and downregulated by thermospermine (Kakehi et al. 2008). This suggests that thermospermine may act in a negative feedback loop that represses auxin-induced xylem differentiation and thermospermine biosynthesis. In fact, a

synthetic proauxin with a delayed release of functional auxin enhances the excess xylem phenotype in *acl5*, but has no obvious effect on xylem differentiation in wild-type seedlings (Yoshimoto et al. 2012). A study in poplar suggests that thermospermine functions are conserved in vascular plants (Milhinhos et al. 2013).

To address the molecular function of thermospermine, we have isolated suppressor mutants named *suppressor-of-acl5* (*sac*), which rescue the *acl5* phenotype in the absence of thermospermine. *SAC51* encodes a basic helix-loop-helix (bHLH) protein. The dominant allele s*ac51-d* has a premature termination codon in one of the upstream open-reading frames (uORFs), introduced by a base substitution in the 5' leader region of the mRNA (Imai et al. 2006). This uORF is well conserved among *SAC51* homologs of different plant species and is classified in homology group 15 (Hayden and Jorgensen 2007). The dominant inheritance of *sac51-d* may be attributed to the removal of the inhibitory effect of this uORF on translation of the main ORF (mORF), resulting in overproduction of the SAC51 protein. Furthermore, *sac52-d*, *sac53-d*, and *sac56-d* represent dominant or semi-dominant alleles of the genes encoding ribosomal protein L10 (RPL10), a receptor for an activated C kinase 1 (RACK1) homolog, which is a component of the small ribosomal subunit, and RPL4, respectively (Imai et al. 2008, Kakehi et al. 2015). In place of thermospermine, these ribosomal mutations may enhance translation of the *SAC51* mORF and stabilize the *SAC51* mRNA by releasing it from uORF-triggered nonsense-mediated mRNA decay (NMD). These findings suggest that *SAC51* plays a role as a target of thermospermine and as a repressor of xylem differentiation. However, it remains unclear how *SAC51* negatively regulates xylem differentiation in a

thermospermine-dependent manner.

A recent study revealed that mutations in conserved uORFs of *SACL1* and *SACL3*, two of the four members of the *SAC51* family, also suppress the *acl5* phenotype (Vera-Sirera et al. 2015). Moreover, *SACL3* expression is activated by the bHLH heterodimers formed by LONESOME HIGHWAY (LHW) with TARGET OF MONOPTEROS5 (TMO5) or TMO5-LIKE1 (T5L1). The resulting SACL3 protein interacts with LHW, thereby limiting the heterodimer formation of LHW-TMO5 and LHW-T5L1 (Katayama et al. 2015, Vera-Sirera et al. 2015). Because *ACL5* expression is directly activated by the LHW-T5L1 heterodimer (Katayama et al. 2015), SACL3 may also play a role in the biosynthesis of, and response to, thermospermine. In this study, with the use of gain- and loss-of-function mutants of these *SAC51* family genes, we show that the function of at least *SAC51* or *SACL3* is required for the repression of both thermospermine biosynthesis and xylem differentiation.

Results

sacl3-d represents a mutation in the conserved uORF of *SACL3*

The *sac57-d* mutation was originally identified as a suppressor of *acl5-1* from an EMS-mutagenized population of *acl5-1* (Imai et al. 2006, Kakehi et al. 2015). As shown below, we found that it represents a dominant allele of *SACL3*, and thus renamed it *sacl3-d*. Mature *acl5-1 sacl3-d* and *acl5-1 sacl3-d/+* plants were morphologically indistinguishable from those of the wild type (Fig. 1A). Genetic mapping, followed by next-generation sequencing of the entire *sacl3-d* genome, revealed a C-to-T base change that resulted in a

Leu-to-Phe substitution in the seventh uORF of *SACL3* (Fig. 1B). This *SACL3* uORF had high sequence similarity to the fourth uORF of *SAC51*, which harbors a point mutation in *sac51-d* (Imai et al. 2006). Such uORFs were conserved within the 5' leader regions of all four members of the *SAC51* family, as well as *SAC51* homologs from different plant species (Fig. 1B). Independently of our study, similar uORF mutations in *SACL3* and in *SAC51* and *SACL1* have been identified as suppressors of *acl5* (Vera-Sirera et al. 2015). From these findings, we concluded that *SACL3* is responsible for the suppressor phenotype. A phylogenetic tree (Supplementary Fig. S1) based on the comprehensive study of Arabidopsis bHLH proteins (Heim et al. 2003, Toledo-Ortiz et al. 2003) placed the SAC51 family close to an atypical bHLH protein subfamily of activation-tagged *bri1* suppressor1-interacting factors (AIFs) (Wang et al. 2009).

In *acl5-1 sacl3-d* seedlings, we examined the expression of the genes that are upregulated in *acl5-1* (Tong et al. 2014). These included *ACL5* from the *acl5-1* allele, *BUSHY AND DWARF2* (*BUD2*), which is also known as *S-ADENOSYLMETHIONINE DECARBOXYLASE4* (*SAMDC4*) and acts as a donor of the aminopropyl group for thermospermine biosynthesis (Ge et al., 2006; Baima et al., 2014), *MONOPTEROS* (*MP*), *TMO5*, *ARABIDOPSIS THALIANA HOMEOBOX8* (*ATHB8*), and *LONELY GUY 4* (*LOG4*). *MP* encodes an auxin response factor that acts as a master regulator for auxin-triggered vascular development (Hardtke and Berleth 1998). *TMO5*, a direct target of MP, encodes a bHLH transcription factor (Schlereth et al. 2010). *ATHB8* is also a target of MP, encoding a class III homeodomain-leucine zipper (HD-ZIP III) protein (Donner et al. 2009). *LOG4*

encodes an enzyme for cytokinin biosynthesis (Kurakawa et al. 2007). The mRNA levels of these genes were restored to wild-type levels in *acl5-1 sacl3-d* (Fig. 1C). We observed that *acl5-1* leaves showed chlorotic symptoms when grown for more than three weeks on MS agar plates, and that these symptoms were also suppressed by *sacl3-d* (Supplementary Fig. S2).

To examine the effect of the *sacl3-d* uORF mutation on mORF translation, we introduced β-glucuronidase (*GUS*) reporter gene constructs into wild-type plants. These constructs were fused to a 2.5-kb genomic fragment containing a 990-bp *SACL3* promoter, and the entire 5' leader region of either wild-type *SACL3* or *sacl3-d* with three introns. The *sacl3-d-GUS* construct yielded between 11 and 23 times more GUS activity than the *SACL3-GUS* construct (Fig. 1D), suggesting that *sacl3-d* causes overproduction of the SACL3 protein.

In contrast to *acl5-1 sacl3-d* double and *sac51-d* single mutants, both of which looked like the wild type, the *sacl3-d* single mutant plants were tiny (Fig. 1E), similar to those observed in a 35S-driven *SACL3* overexpression line (Vera-Sirera et al. 2015). The *acl5-1 sac51-d sacl3-d* triple mutant seedlings were also tiny (Fig. 1E). This suggests that overproduction of SACL3 has an inhibitory effect on growth in the presence of thermospermine.

Responses of *SAC51* family genes to thermospermine

The *SAC51* family mRNAs have long 5' leader regions with multiple uORFs (Fig. 2A). Our previous studies have suggested that thermospermine alleviates the inhibitory effect of

uORFs on translation of the main ORF of *SAC51*, and stabilizes the *SAC51* mRNA (Kakehi et al. 2015). We thus examined the effect of thermospermine on the expression of *SAC51* family genes. The mRNA levels of *SAC51* and *SACL1* were increased upon treatment with thermospermine for 24 h, but those of *SACL2* and two alternatively spliced isoforms of *SACL3* were decreased (Fig. 2B); a result similar to that in *acl5-1* (Kakehi et al. 2010). The *GUS* constructs with fusions to each gene promoter and 5' leader were also tested for their responses to thermospermine. Similar to transgenic lines containing a *SAC51-GUS* construct (Kakehi et al. 2008), the seedlings harboring *SACL1-GUS* constructs showed several-fold increases in GUS activity after 24 h treatment with thermospermine. However, those with *SACL2-GUS* or *SACL3-GUS* showed no significant response to thermospermine (Fig. 2C).

sac51-1 sacl3-1 has an increased level of thermospermine

To elucidate the functions of each member of the *SAC51* family, T-DNA insertion mutants of each gene were obtained and named *sac51-1*, *sacl1-1*, *sacl2-1*, and *sacl3-1*, respectively (Fig. 2A). qRT-PCR data showed that, whereas *sacl1-1* had increased accumulation of *SACL1* mRNA, *sac51-1*, *sacl2-1*, and *sacl3-1* had no detectable levels of the mRNA for the corresponding mORFs (Supplementary Fig. S3). This suggested that *sac51-1*, *sacl2-1*, and *sacl3-1* were loss-of-function mutants, but *sacl1-1* was not. Further qRT-PCR analysis with 5'-specific primers of each mRNA revealed that *SACL2* expression was enhanced in *sacl2-1* (Supplementary Fig. S3), suggesting negative autoregulation of *SACL2*.

Because each single mutant showed normal morphology, we generated multiple

mutants by genetic crosses. All genotypes were confirmed by PCR (Supplementary Fig. S4). Among other double mutant combinations, *sac51-1 sacl3-1* exhibited a slight but significant reduction in plant height (Fig. 3A). Furthermore, while *sac51-1 sacl1-1 sacl3-1* showed a similar growth phenotype to *sac51-1 sacl3-1* (Supplementary Fig. S5), *sac51-1 sacl2-1 sacl3-1* and the *sac51-1 sacl1-1 sacl2-1 sacl3-1* quadruple mutant, hereafter referred to as *sacs*, exhibited a more severe phenotype (Fig. 3B; Supplementary Fig. S5), suggesting an additional effect of *sacl2-1* on the phenotype. Under microscopy, we observed that lignified xylem vessels were more developed in *sac51-1 sacl3-1* and *sacs* hypocotyls than in the wild-type hypocotyl, a phenotype reminiscent of *acl5-1* (Fig. 3C). In *acl5-1*, vein development in cotyledons is greatly enhanced in the presence of a synthetic proauxin, isooctyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D IOE) (Yoshimoto et al. 2012). This phenotype was also mimicked in *sac51-1 sacl3-1* and *sacs* grown with 2,4-D IOE (Fig. 3D). Most importantly, measurement of tetraamine levels by HPLC revealed that mutants homozygous for both *sac51-1* and *sacl3-1*, in particular *sacs*, had significantly higher levels of thermospermine than the wild type, and even more than *pao5-2* (Fig. 3E). *PAO5* encodes a polyamine oxidase that predominantly catalyzes thermospermine degradation (Ahou et al. 2014, Kim et al. 2014). These results suggest that members of the *SAC51* family function redundantly and that the presence of *SAC51* or *SACL3* is required for the repression of thermospermine biosynthesis and xylem differentiation.

sac51-1 sacl3-1 is insensitive to thermospermine

Next, we examined the response of mutant seedlings to exogenously supplied thermospermine. When grown on MS agar plates supplemented with 100 μM thermospermine, wild-type seedlings show reduced leaf and root growth (Fig. 4A-C; Supplementary Fig. S6 for the number of lateral roots). Root sections revealed that thermospermine greatly repressed xylem differentiation in the wild type (Fig. 4D). Therefore, the inhibitory effect of thermospermine on growth may be caused by the repression of xylem differentiation in the root, and consequently the reduction or loss of function of xylem vessels. Double mutants of *sac51-1 sacl1-1* and *sac51-1 sacl2-1* as well as each single mutant showed similar growth inhibition. By contrast, *sac51-1 sacl3-1* and *sacs* seedlings were insensitive to thermospermine (Fig. 4A-C) and showed no repression of xylem differentiation in the root (Fig. 4D). This again suggests that at least one of *SAC51* and *SACL3* is required to elicit a response to thermospermine. Expression levels of the genes upregulated in *acl5-1* and their responses to thermospermine were examined in *sacs* seedlings. Among those examined, mRNA levels of *ACL5*, *BUD2*, and *LACCASE6* (*LAC6*) in *sacs* were about two-fold higher than in the wild type, although those of *MP*, *TMO5*, *ATHB8*, and *LOG4* were not significantly affected (Fig. 4E). The mRNA levels of *ACL5*, *BUD2*, *LAC6*, *MP*, and *LOG4* in *sacs* showed about a two-fold reduction after 24 h treatment with thermospermine, but those of *TMO5*, *T5L1*, and *ATHB8* were not altered (Fig. 4E). These results indicate that *sacs* remained responsive to thermospermine, possibly because *sacl1-1* is not a null allele.

***acl5-1 sacl3-1* shows a very tiny plant size**

To further clarify the relationship between *ACL5* and the *SAC51* family, double or multiple mutants of *acl5-1* with each T-DNA mutant were generated. While *acl5-1 sac51-1*, *acl5-1 sacl1-1*, and *acl5-1 sacl2-1* were indistinguishable from *acl5-1*, *acl5-1 sacl3-1* plants had very small leaves (Fig. 5A), were tiny in size, and were sterile (Fig. 5B). Quintuple *acl5-1 sacs* mutants also showed a tiny-plant phenotype (Fig. 5B). This synergistic phenotype suggested that *ACL5* and *SACL3* function in parallel and interrelated pathways. Examination of root sections revealed that *acl5-1 sacs* tiny seedlings showed normal development of vascular cell files while tiny seedlings of *sacl3-d* and *sac51-d sacl3-d acl5-1* (Fig. 1E) had reduced number of vascular cells with no apparent xylem vessel elements (Supplementary Fig. S7). The latter is reminiscent of 35S-driven *SACL3* overexpression plants (Katayama et al. 2015, Vera-Sirera et al. 2015) and the higher-order *tmo5* and *lhw* loss-of-function mutants (De Rybel et al. 2013, Ohashi-Ito et al. 2013a). SACL3 has been shown to interfere with the bHLH heterodimer formation of LHW-TMO5 and LHW-T5L1 (Katayama et al. 2015, Vera-Sirera et al. 2015). Thus we finally examined steady-state mRNA levels of *SAC51* family genes in *acl5-1* and *lhw*. Only the *SACL3* mRNA level was significantly increased in *acl5-1* and reduced in *lhw* (Fig. 5C). We also confirmed that wild-type seedlings grown in the presence of thermospermine had greatly reduced levels of *SACL2* and *SACL3* mRNA (Fig. 5C). According to expression analysis in the root using the GFP reporter, while *SAC51* is broadly expressed in the vascular cylinder, expressions of *SACL1*, *SACL2*, and *SACL3* are restricted to protophloem poles, cambial cells, and both xylem and pericycle, respectively (Vera-Sirera et al. 2015). Therefore, the increase in the *SACL3* mRNA level in *acl5-1* and the reduction in *SACL2* and

SACL3 mRNA levels by thermospermine may reflect thermospermine-dependent repression of xylem differentiation.

Discussion

Our results revealed that one of *SAC51* or *SACL3* is required for thermospermine-dependent negative control of both xylem differentiation and thermospermine biosynthesis in Arabidopsis. Taken together with published data, we propose a model for a thermospermine-mediated regulatory network (Fig. 6). *ACL5* expression has been shown to be under the direct control of ATHB8 (Baima et al. 2014). *ATHB8* is a direct target of MP, an auxin response factor essential for the specification of vascular stem cells (Donner et al. 2009). *MP* also directly regulates expression of *TMO5* and *T5L1* (Schlereth et al. 2010). *LHW* is required for correct expression patterns of *MP* and *ATHB8* (Ohashi-Ito et al. 2013b). Therefore, downregulation of these genes by thermospermine might be attributed to the interfering effect of SAC51 family proteins on the function of LHW to heterodimerize with TMO5 or T5L1. In turn, these heterodimers induce expression of *SACL3* and *ACL5* (Katayama et al. 2015), while also inducing a cytokinin biosynthetic gene, *LOG4*, and *AHP6*, which suppresses cytokinin signaling, for maintaining growth and patterning of the embryonic vascular tissues (Ohashi-Ito and Bergmann 2007, Ohashi-Ito et al. 2014). In line with the model, we found a reduction of vascular cell files with no apparent xylem vessels in the root of the wild type grown with thermospermine (Fig. 4D) and that of *sacl3-d* and *sac51-d sacl3-d acl5-1* (Supplementary Fig. S7).

In contrast to *SAC51* and *SACL1*, *SACL2* and *SACL3* were not translationally upregulated by thermospermine under our experimental conditions. The transgenic lines used in our work produce endogenous thermospermine, and it is possible that the tissues expressing *SACL2* and *SACL3* already contain sufficient thermospermine that exogenous thermospermine is no longer effective to induce the reporter activity. Levels of *SACL2* and *SACL3* mRNA were rather reduced by 24 h treatment with thermospermine (Fig. 2B), suggesting that these mRNAs are not stabilized by thermospermine. On the other hand, *SAC51* and *SACL1* mRNAs may be stabilized by thermospermine probably because they are released from uORF-triggered NMD (Kakehi et al. 2015).

Given that major targets of thermospermine are *SAC51* and *SACL1*, the *acl5* phenotype was expected in double knockouts of *SAC51* and *SACL1*. However, *sac51-1 sacl1-1* showed a normal growth phenotype. This may be because *sacl1-1* is not a loss-of-function mutant but rather has an increased level of the mRNA encompassing the *SACL1* coding region (Supplementary Fig. S3). The normal sensitivity of *sac51-1 sacl1-1* to thermospermine (Fig. 4C) may also be caused by a weak *sacl1-1* allele. The reason *sacs* displays a weak dwarf phenotype with only a moderate or no increase in the expression of the genes upregulated in *acl5-1* (compare Fig. 1C and 4E), yet still responds to thermospermine (Fig. 4E), may again be because of a weak *sacl1-1* allele. In addition, vein development in cotyledons was slightly enhanced in *sac51-1 sacl3-1* and *sacs* and further enhanced by 2,4-D IOE, as in *acl5-1* (Fig. 3D). It is thus possible that *sacs* mimics a predicted weak *acl5* phenotype. Surprisingly, however, *acl5-1 sacl3-1* and *acl5-1 sacs* displayed the tiny-plant

phenotype (Fig. 5B). Given that *acl5-1* is equivalent to loss of function of *SAC51* and *SACL1*, this phenotype might represent the one predicted to result from the complete loss of the *SAC51* family. Because *sacl2-1* had some additional effect on the growth phenotype of *sac51-1 sacl3-1* (compare *sac51-1 sacl3-1* and *sacs* in Fig. 3; Supplementary Fig. S5) but no visible effect on the phenotype of *acl5-1* (Fig. 5A), *SACL2* might play a subsidiary role in the module. On the other hand, the importance of *SACL1* remains unclear. Isolation of a null mutant of *SACL1* is required for further study.

The apparent contrast of the normal growth of *sac51-d*, *sac51-d acl5-1*, and *sacl3-d acl5-1*, and the tiny-plant phenotype observed in *sacl3-d* and *sac51-d sacl3-d acl5-1* (Fig. 1E), namely, the fact that the phenotype is manifested only by *sacl3-d* in the presence of *SAC51* (whose translation is enhanced by thermospermine but not vice versa) also suggests the parallelism of *ACL5* (thermospermine)-dependent *SAC51* and *SACL3*. No apparent xylem formation in the root of the tiny seedlings may phenocopy the effect of excess thermospermine on wild-type seedlings (Fig. 4D) and might cause critical nutritional deficiency leading to the tiny growth. The tiny-plant phenotype is also phenocopied by the higher-order *tmo5* and *lhw* loss-of-function mutants (De Rybel et al. 2013, Ohashi-Ito et al. 2013a, Vera-Sirera et al. 2015). Based on these results, it is suggested that SAC51 and SACL3 act in a nested feedback loop in which thermospermine-induced SAC51 interferes with the function of LHW-TMO5/T5L1 that activates expression of *SACL3* and *ACL5* (Fig. 6). According to the model, the tiny plant phenotype of *acl5-1 sacl3-1* (Fig. 5B) might be resulted from overactivation of LHW-TMO5/T5L1, although its direct cause remains to be

addressed.

Compared with *pao5*, *sacs* accumulates a significantly higher level of thermospermine. *pao5* is defective in thermospermine degradation but accumulates only about two-fold higher levels of thermospermine (Fig. 3E) (Kim et al. 2014). This can be explained by negative feedback control of *ACL5* expression, which should function in *pao5*, and possible translocation of excess thermospermine in *sacs*, which could no longer be catabolized by vascular-specific PAO5 (Kim et al. 2014).

Considering the different response to thermospermine of *SAC51* family genes, which commonly contain conserved uORFs, it is possible that these conserved uORFs have no direct influence on the plant's response to thermospermine, yet do have an inhibitory effect on mORF translation. Intracellular polyamines generally exist in a polyamine-RNA complex (Igarashi and Kashiwagi 2010). Spermine binds a bulged region of the double-stranded structure within specific RNA molecules (Igarashi and Kashiwagi 2000, Ennifar and Dumas 2006). Thermospermine might also interact with a bulged structure that is presumably formed within the 5' leader sequences of *SAC51* and *SACL1*. In this case, thermospermine might act as a riboswitch ligand that alters the RNA secondary structure and allows the scanning small ribosomal subunit to reach the mORF initiation codon. Riboswitches have been rarely identified in plants. One example is a thiamine pyrophosphate (TPP) riboswitch in the 3' untranslated region of the thiamine-biosynthetic gene (Bocobza et al. 2007, Wachter et al. 2007). Another possibility cannot be excluded that the nascent polypeptides translated from the conserved uORFs of *SAC51* and *SACL1* interact with

thermospermine. In vertebrates, high levels of polyamines trigger translational repression of the gene for *S*-adenosylmethionine decarboxylase by causing ribosome stalling at the termination of a conserved uORF encoding a hexapeptide, MAGDIS (Ivanov et al. 2010). In Arabidopsis, *SAMDC1* has two overlapping uORFs. Although the precise mode of action is unknown, the second and longer uORF is preferentially translated by scanning ribosomes at high levels of polyamines, and prevents them from reinitiating translation of the mORF (Hanfrey et al. 2005). This uORF is also conserved in other members of the *SAMDC* family, except for *BUD2* (*SAMDC4*), and in different plant species (Jorgensen and Dorantes-Acosta 2012). Another conserved uORF is present in the Arabidopsis genes for polyamine oxidase, *PAO2*, *PAO3*, and *PAO4* (Guerrero-González et al. 2014), suggesting a close relationship between polyamines and conserved uORFs. Further detailed examination of the responsiveness of each 5' leader region of *SAC51* family genes to thermospermine will be necessary to elucidate how translation is enhanced by thermospermine.

In conclusion, we found that thermospermine responses involve two parallel and interconnected signaling pathways mediated by *SAC51* and *SACL3*. *ACL5* and *SACL3* are expressed in xylem precursor cells under the direct control of LHW-T5L1 (Katayama et al. 2015) while *SAC51* and *SACL1* are expressed throughout the vascular cylinder and in protophloem poles, respectively (Vera-Sirera et al. 2015). Given the result that thermospermine predominantly enhances translation of *SAC51* and *SACL1*, it is reasonable to assume that only *SAC51* and *SACL1* have evolved or retained the ability to respond to thermospermine, which diffuses from xylem precursor cells. The tiny-plant phenotype of

acl5-1 sacl3-1 clearly indicates the importance of these signaling pathways in vascular development but the causal relationship between the mutations and the phenotype needs further investigation.

Materials and Methods

Plant material, growth conditions, and chemicals

Arabidopsis thaliana Col-0 and L*er* accessions were used as wild-type strains. *acl5-1* (Hanzawa et al. 1997) and *sac51-d* (Imai et al. 2006) are as described. *sacl3-d* was originally identified as *sac57-d* by screening an EMS-mutagenized population of *acl5-1* (L*er*) seeds for suppressor mutants (Kakehi et al. 2015). T-DNA insertion lines, *sac51-1* (SALK_107954), *sacl1-1* (SAIL_743_E04), *sacl3-1* (SALK_147291), *pao5-2* (SALK_053110C), and *lhw* (SALK_079402C), were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). *sacl2-1* (GK-033B04-026598) was obtained from the GABI-KAT T-DNA insertion collection (http://www.gabi-kat.de).

Seeds were surface-sterilized in bleach solution containing 0.01% (v/v) Triton X-100 for 3 min, washed 3 times with sterile water, and sown onto rockwool cubes or Murashige and Skoog (MS) medium containing 3% sucrose and 0.8% agar. Plants were grown under 16 h fluorescent light at 22°C. For thermospermine treatment, seedlings were incubated for 24 h in liquid MS medium with 100 μM thermospermine-4HCl, which was synthesized by Masaru Niitsu (Niitsu et al. 1992). 2,4-D IOE was synthesized by by Ken-Ichiro Hayashi (Hayashi et al. 2008) and dissolved in dimethyl sulfoxide before use.

Next-generation sequencing and genotyping

Genetic mapping of the *sacl3-d* allele was performed as described (Kakehi et al. 2015). The mutation was identified by next-generation sequencing technology (Uchida et al. 2011). Briefly, genomic DNA extracted from *sacl3-d* was fragmented to approximately 150 bp using a Covaris S2 DNA sonicator (Covaris, Woburn, MA), end-repaired, adenylated, and ligated to adapters. The purified libraries were amplified by PCR, quantified with the High Sensitivity DNA assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and sequenced using an Illumina Genome Analyser IIx (Illumina, San Diego, CA).

Genotypes of *acl5-1*, *sac51-d* and *sacl3-d* were determined by the dCAPS method (Neff et al. 1998). Genotypes of T-DNA insertion alleles were confirmed by PCR using respective gene- and T-DNA-specific primers. Primers and restriction enzymes used are listed (Supplementary Table S1).

T-DNA construction and plant transformation

The *GUS* gene construct fused to the *SAC51* promoter and 5' leader has been described previously (Imai et al. 2006). Other *GUS* fusions were constructed by cloning each gene promoter fragment with 5' leader that was amplified by PCR with gene-specific primers (Supplementary Table S2) into pGEM-T Easy vector (Promega, Madison, WI) and transferring into a Ti-plasmid vector pBI101 (Clontech, Palo Alto, CA).

Ti plasmid constructs were introduced into *Agrobacterium tumefaciens* C58C1 by

electroporation. Transgenic Arabidopsis plants were generated by the floral dip method (Clough and Bent 1998). More than twelve lines were obtained for each construct and the data are shown for one representative line unless otherwise stated.

Microscopy

Samples were cleared with chloral hydrate as described (Yoshimoto et al. 2012) and observed under a light microscope equipped with Nomarski DIC optics. For tissue sections, samples were fixed, embedded into Technovit 7100 resin (TAAB laboratories, Reading, UK), sectioned into 10 μm-thick slices, and stained with toluidine blue.

GUS assays

The GUS activity was fluorometrically measured as described previously (Jefferson et al. 1987) for each transgenic line of 7-day-old seedlings. The fluorescence was measured with an RF-5300PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). Total protein content was measured by using the Bradford assay (Bio-Rad, Hercules, CA).

qRT-PCR

Total RNA was isolated from seedlings according to the SDS-phenol method and reverse-transcribed using PrimeScript RT reagent Kit (Takara, Kyoto, Japan) with the oligo dT primer. The resulting first-strand cDNA was used for real-time PCR with gene-specific primers (Supplementary Table S3). PCR reactions were performed using KAPA SYBR FAST

qPCR Kit (KAPA Biosystems, Boston, MA) and the DNA Engine Opticon2 System (Bio-Rad). *ACTIN8* (At1g49240) was used as an internal standard in the reactions.

HPLC

Polyamines were extracted from seedlings and benzoylated as described previously (Tong et al. 2014). Benzoylated polyamines were analyzed using an Agilent 1120 Compact LC HPLC system with a TSKgel ODS-80Ts reverse-phase column (Toso, Tokyo, Japan). The elution was performed with 42% (v/v) acetonitrile at a flow rate of 0.5 mL/min for 50 min and monitored by UV absorbance at 254 nm.

Chlorophyll assays

Chlorophyll was extracted from 50 mg of seedlings in 1 mL of *N*,*N*-dimethyl formamide at 4°C overnight in the dark and assayed as described (Porra et al. 1989).

Funding

This work was supported in part by Grants-in-Aid for Scientific Research [No. 26113516, No. 16H01245] from the Japan Society for the Promotion of Science (JSPS) to TT.

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgements

We thank Prof. Masaru Niitsu for providing thermospermine, Prof. Ken-Ichiro Hayashi for providing 2,4-D IOE, and Prof. Dolf Weijers for sharing unpublished data and for helpful discussion. The authors declare no competing or financial interests.

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Figure legends

Fig. 1 Characterization of *sacl3-d*. (A) Appearance of 35-day-old plants of *acl5-1*, *acl5-1 sacl3-d/+*, *acl5-1 sacl3-d*, and the wild type Landsberg *erecta* (L*er*). Scale bar: 2 cm. (B) Alignment of the conserved uORFs of the homology group 15 (Jorgensen and Dorantes-Acosta, 2012). The deduced polypeptide sequences of all members of the Arabidopsis *SAC51* family and those of *SAC51*-like genes of *Populus euphratica* (XP_011043085) and *Brachypodium distachyon* (XP_010232047) are shown. An amino acid substitution in *sacl3-d* is indicated with a nucleotide substitution in parentheses. (C) Relative mRNA levels of *ACL5*, *BUD2*, *MP*, *TMO5*, *ATHB8*, and *LOG4* in the wild type (Wt), *acl5-1*, and *acl5-1 sacl3-d*. Total RNA was prepared from 7-day-old seedlings and analyzed by quantitative real-time RT-PCR. All mRNA levels are relative to those in the wild type. (D) Effect of *sacl3-d* in the *SACL3* promoter-5'-*GUS* fusion on the GUS activity. The data on four independent lines with the wild-type 5'-*GUS* (Wt) fusion and those with the *sacl3-d* 5'-*GUS* (*sacl3-d*) fusion are shown relative to the average of wild-type 5'-*GUS* lines set as 1. (E) Appearance of 30-day-old plants of *sac51-d*, *sacl3-d*, and *acl5-1 sac51-d sacl3-d*. Scale bar: 1 cm. Values in (C) and (D) are mean ± SD. (n = 3, **P*<0.05). Asterisks indicate Student's

t-test significant differences from the mean value of the wild type.

Fig. 2 Thermospermine response of the *SAC51* family. (A) Genomic structure of all members of the *SAC51* family based on data from the Arabidopsis Information Resource (http://www.arabidopsis.org/). Boxes indicate exons and bars indicate promoter regions and introns. Black and half-width gray areas represent mORFs and uORFs, respectively. Locations of the mutations are show by arrowheads. Pairs of arrows indicate primers used for RT-PCR. (B) Relative mRNA levels of *SAC51*, *SACL1*, *SACL2*, and two alternatively spliced isoforms of *SACL3* in the wild type. Seven-day-old seedlings were incubated in the MS solution with no or 100 μM thermospermine for 24 h. All mRNA levels are relative to those in seedlings treated with no thermospermine. Values are mean ± SD. (n = 3, **P*<0.05). Asterisks indicate Student's *t*-test significant differences. (C) Effect of 24-h treatment with thermospermine on the GUS activity of 7-day-old seedlings carrying each promoter-5'-*GUS* fusion. Values are mean ± SD. (n = 3, **P*<0.05). Asterisks indicate Student's *t*-test significant differences.

Fig. 3 A quadruple mutant mimics *acl5*. (A) Comparison of plant height of the wild type (Wt (Col-0)) and each mutant grown for 40 days. Values are mean ± SD. (n = 8). Asterisks indicate significant differences from the wild type (**P* < 0.05; ***P* < 0.01; ****P* < 0.001 by Student's *t*-test). (B) Appearance of wild-type, *sac51-1 sacl3-1*, and *sac51-1 sacl1-1 sacl2-1 sacl3-1* (*sacs*) plants grown for 35 days. (C) Vascular phenotype in the hypocotyl of

7-day-old seedlings of the wild type, *sac51-1 sacl3-1*, *sacs*, and *acl5-1* in the Col-0 background. Scale bar: 0.5 mm. (D) Effect of 2,4-D IOE on vein development in cotyledons of the wild type, *sac51-1 sacl3-1*, *sacs*, and *acl5-1*. Plants were grown for 7 days in the MS solution with no (-) or 3 μM (+) 2,4-D IOE. In (C) and (D), samples were cleared by chloral hydrate and autofluorescence of lignin and phenolic compounds was visualized by a polarizing plate under microscope with DIC optics. (E) Tetraamine content in the wild type and each mutant. Polyamines were extracted from 7-day-old seedlings and analyzed by HPLC. Values are mean ± SD. (n = 3). Asterisks indicate significant differences from the wild type (*P < 0.05; **P < 0.01 by Student's *t*-test).

Fig. 4 *sac51-1 sacl3-1* is insensitive to thermospermine. (A) Effect of thermospermine on the fresh weight of aerial part of seedlings. Plants were grown for 10 days on the MS agar plate with no or 100 μM thermospermine. (B) Effect of thermospermine on the root length of seedlings. Plants were grown for 10 days on the MS agar plate with no or 100 μM thermospermine. (C) Effect of thermospermine on the seedling growth. Plants were grown for 10 days on the MS agar plate with 100 μM thermospermine. (D) Vascular phenotype in the root of the wild type, *sacl3-1*, *sac51-1 sacl3-1*, and *sacs*. Plants were grown for 10 days on the MS agar plate with no or 100 μM thermospermine. Scale bar: 20 μm. (E) Relative mRNA levels of *ACL5*, *BUD2*, *LAC6*, *MP*, *TMO5*, *ATHB8*, and *LOG4* in the wild type and *sacs*. Ten-day-old seedlings were incubated in the MS solution with no or 100 μM thermospermine for 24 h. All mRNA levels are relative to those in wild-type seedlings treated with no

thermospermine. Values in (A), (B), and (E) are mean ± SD. (n = 3, *P<0.05). Asterisks indicate Student's *t*-test significant differences.

Fig. 5 Interrelationship between *ACL5*, *LHW*, and the *SAC51* family. (A) Seedling phenotypes of *acl5-1 sac51-1*, *acl5-1 sacl2-1*, and *acl5-1 sacl3-1*. Seven-day-old seedlings are shown. Scale bar: 5 mm. (B) Phenotypes of 30-day-old plants of *acl5-1 sacl3-1* and *acl5-1 sacs*. Scale bar: 5 mm. (C) Relative mRNA levels of *SAC51*, *SACL1*, *SACL2*, and *SACL3* in wild-type, *acl5-1*, and *lhw* seedlings. Plants were grown for 10 days on the MS agar plate with no (Wt, *acl5-1*, and *lhw*) or 100 μM thermospermine (Wt+Tspm). All mRNA levels are relative to those in wild-type seedlings grown with no thermospermine. Values are mean ± SD. (n = 3, *P<0.05). Asterisks indicate Student's *t*-test significant differences.

Fig. 6 A model of the *SAC51* family-mediated regulatory module for auxin-induced xylem formation in the root. The auxin signaling for xylem formation shown here is not comprehensive (for details see e.g. Miyashima et al. 2013, Růžička et al. 2015). PIN1 is an auxin efflux carrier that controls polar auxin transport (Scarpella et al. 2006). Dashed lines indicate protein-protein interactions.