A study on the mode of action of thermospermine in stem elongation of *Arabidopsis thaliana*.

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ABBREVIATIONS

2,4-D : 2,4-dichlorophenoxyacetic acid (a synthetic auxin)
2,4-D-IOE : 2,4-D isooctyl ester
ACL5 (ACAULIS5) : thermospermine synthase
ACT8 : actin 8 (internal control for real-time PCR)
ADC : arginine decarboxylase
AENSD : aminoethyl norspermidine
AIH : agmatine iminohydrolase
APNSD : aminopentyl norspermidine
ATHB8 : HD-ZIP III transcription factor regulating the differentiation of xylem vessels
bud2 : mutation of the *SAMDC4* gene
CPA : N-carbamoylputrescine amidohydrolase
dcSAM : decarboxylated *S*-adenosyl methionine
GUS : beta-glucuronidase
HCP : homocaldopentamine
NGD : nogo-mediated mRNA decay
NMD : nonsense-mediated mRNA decay
NSPD : norspermidine
NSPM : norspermine
ODC : ornithine decarboxylase
PAO : polyamine oxidase
PHB : HD-ZIP III transcription factor regulating the differentiation of xylem vessels
PMT : putrescine N-methyltransferase
PTC : premature termination codon
PUT : putrescine
sac mutants : suppressors of *acaulis5*
SAC51 : bHLH transcription factor
SAC52 : ribosomal protein L10 (RPL10A)
SAC53 : receptor for activated C kinase 1 (RACK1A), component of 40S ribosomal subunit
SAC56 : ribosomal protein L4 (RPL4A)
SACL1~3 : *SAC51-like* genes
SAMDC1~4 : *S*-adenosyl methionine decalboxylases
SPD : spermidine
SPDS : spermidine synthase
SPM : spermine
SPMS : spermine synthase
TSPM : thermospermine
UBQ10 : ubiquitin 10 (internal control for real-time PCR)
uORF : upstream open reading flame
UPF1 : regulator of nonsense transcripts 1, essential factor in NMD
UPF3 : regulator of nonsense transcripts 3, essential factor in NMD
VND7 : NAC transcription factor regulating the differentiation of xylem vessels

GENERAL INTRODUCTION

The polyamines putrescine, spermidine, and spermine are ubiquitous polycationic compounds, and play important roles in regulating transcription, RNA turnover, translation, and in controlling cell growth, metabolism, and development. In plants, polyamines are involved in embryogenesis, plant stress responses and organ development (Kusano et al., 2008; Takahashi and Kakehi, 2009). The *acaulis5* (*acl5*) mutant shows severe dwarf phenotype due to excess xylem differentiation and reduced cell elongation (Hanzawa et al., 1997). Previous studies suggested that *ACL5* encodes spermine synthase (Hanzawa et al., 2000). Later, however, a study by the other group indicated that an *ACL5* homolog in the diatom, *Thalassiosira pseudonana*, encodes thermospermine synthase (Knott et al., 2007). In my study in the undergraduate program, I revealed that *Arabidopsis thaliana ACL5* encodes thermospermine synthase and exogenous treatment of *acl5* plants with thermospermine restores its dwarf phenotype (Kakehi et al., 2008). Thermospermine is a structural isomer of spermine (Fig. 1), and was first discovered in an extremely thermophilic bacterium, *Thermus thermophilus* (Oshima et al., 1979). *ACL5* orthologs are conserved in some bacteria and archaea and ubiquitously in the plant kingdom (Takahashi and Kakehi, 2010; Takano et al., 2012; Fig. 2). However, molecular functions of thermospermine remain unclear.

To reveal the functions of thermospermine, suppressor mutants that restore the dwarf phenotype of *acl5*, named *suppressors of acaulis5* (*sac*), were isolated. The *sac51-d* dominant mutant fully restores the *acl5* phenotype and *SAC51* encodes a basic helix-loop-helix (bHLH) transcription factor (Imai et al., 2006). *SAC51* mRNA contains five short upstream open reading frames (uORFs) within the 5' leader sequence and the *sac51-d* allele has a single base substitution that introduces a premature termination codon in the 4th uORF. This mutation has been shown to increase the translation efficiency of the *SAC51* main ORF (Imai et al., 2006), suggesting that SAC51 overproduction uncouples the stem growth from its control by thermospermine. Furthermore, the gene responsible for *sac52-d* has been shown to encode a ribosomal protein RPL10, which is a component of the 60S large ribosomal subunit, and *sac52-d* also increases the translation efficiency of the *SAC51* main ORF (Imai et al., 2008). On the other hand, a previous study has found that *sac53-d* has a single base substitution in a gene encoding a receptor for activated C kinase1 (RACK1), although genetic complementation experiments have remained to be performed (Kawano, unpublished). RACK1 is a component of the 40S small ribosomal subunit.

In this study, I first focused on the structural difference between thermospermine and spermine and determined molecular properties required for the biological activity in thermospermine. Secondly, to reveal the mode of action of thermospermine in plant growth, I performed further genetic experiments on *sac* mutants and revealed that *SAC53* indeed encodes RACK1 while the gene responsible for *sac56-d* encodes RPL4. Finally, I addressed the relation between the function of these ribosomal components and thermospermine-dependent uORF-mediated control of *SAC51* translation.

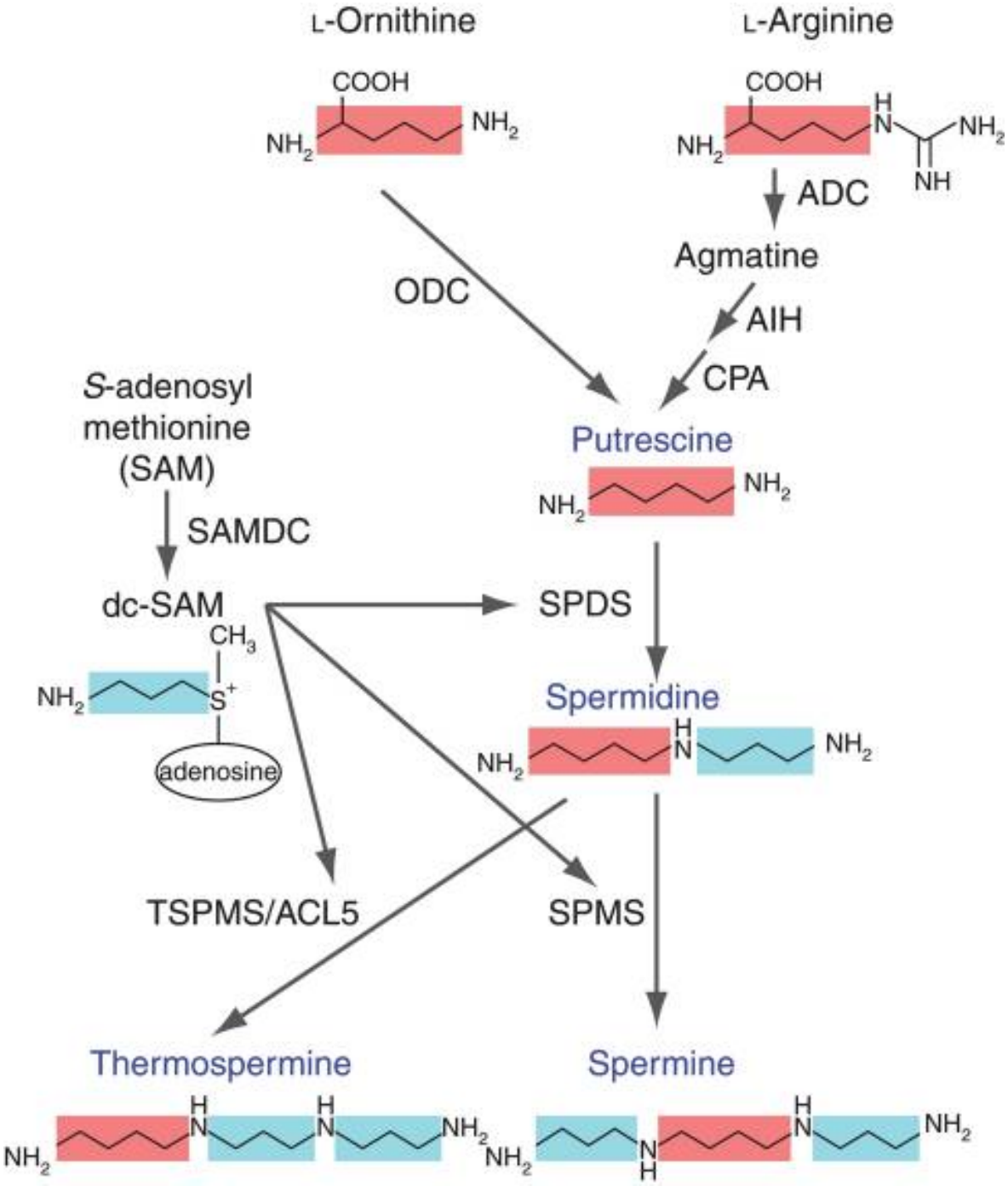

Fig. 1

Biosynthetic pathways of polyamines in plants.

Abbreviations: ACL5, ACAULIS5; ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, N-carbamoylputrescine amidohydrolase; ODC, ornithine decarboxylase; SAMDC, *S*-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; TSPMS, thermospermine synthase.

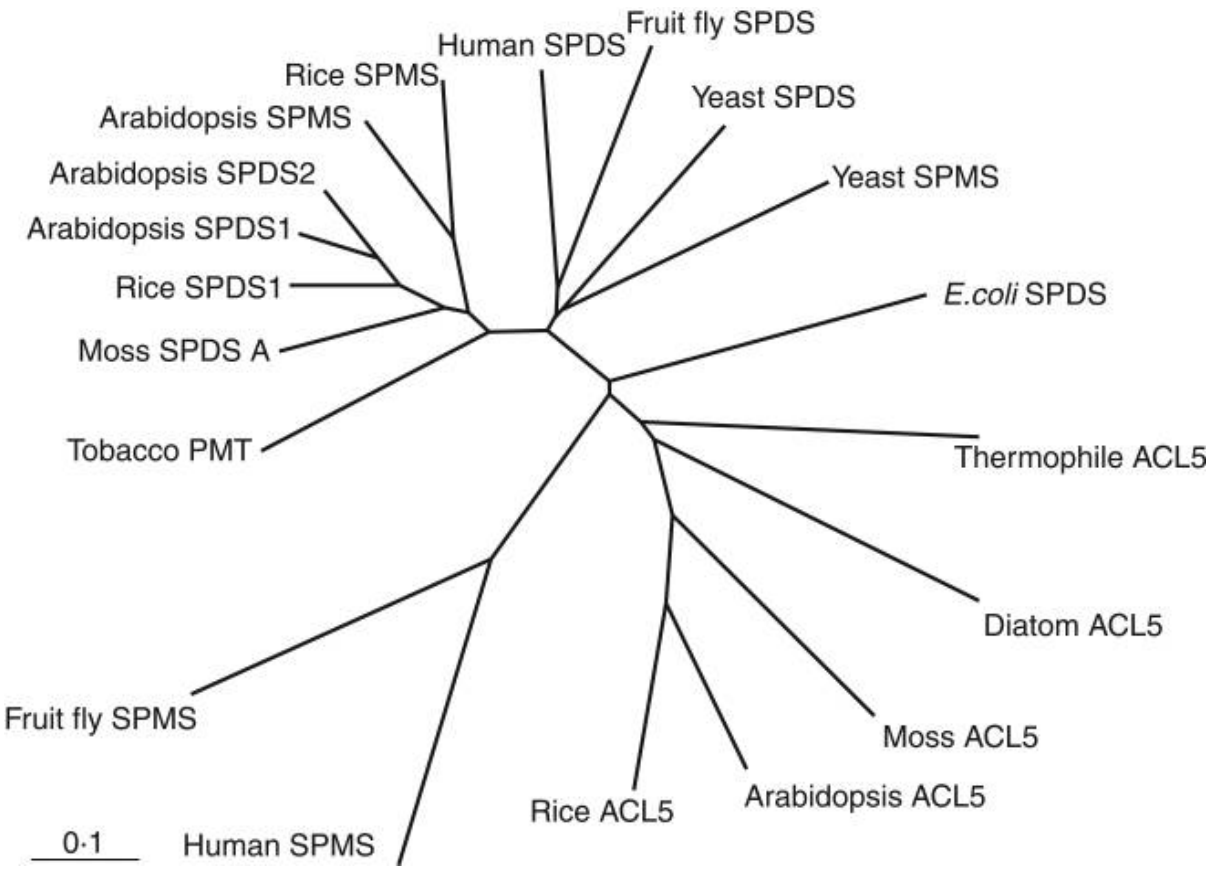

Fig. 2

Molecular phylogenetic tree of the amino acid sequences of spermidine/spermine synthase-related proteins.

The full-length amino acid sequences were aligned by using Clustal W and the tree was drawn by the neighbor-joining method with the software TreeView. *ACL5* represents thermospermine synthase or its putative orthologs. Thermophile, Diatom, and Moss represent *Thermus thermophilus, Thalassiosira pseudonana,* and *Physcomitrella patens*, respectively.

Abbreviations: PMT, putrescine N-methyltransferase

MATERIALS AND METHODS

Chemicals

All polyamines used in this study were hydrochloride salts. Spermine, spermidine, and norspermidine were purchased from Sigma-Aldrich. Thermospermine and uncommon polyamines were provided by Masaru Niitsu, Josai University, Japan (Niitsu et al., 1992) and Kazuei Igarashi, Chiba University, Japan (Oshima, 1983). 2,4-D-IOE is as described in Yoshimoto et al. (2012).

Plant material and growth condition

The *A. thaliana* mutant strains *acl5-1* and *spms-1* were as previously described (Hanzawa et al., 1997; Imai et al., 2004). *sac52-d acl5-1* and *sac53-d acl5-1* mutants were previously isolated as suppressors of *acl5-1* (Imai et al. 2006). The *sac56-d* mutant was identified from additional screening for suppressor mutants of *acl5-1* (Kawano, unpublished). *rack1a-1* and *rack1a-2* are as described in Chen et al. (2006). *bud2-2* is as described in Ge et al. (2006). *upf1-1* and *upf3-1* are as described in Yoine et al. (2006) and Hori et al. (2005), respectively.

Plants were grown on rock wool or Murashige-Skoog (MS) medium supplemented with 3% sucrose at 22°C under continuous light. For the analysis of mRNA stability, 10-day-old plants grown on liquid MS medium were treated with 0.6 mM cordycepin for the indicated period.

For daily treatment of plants with a polyamine, 40 µl of the respective 0.1 mM polyamine solution was added to the shoot apex of *acl5-1 spms-1* from 10 days after germination.

Mapping and genotyping

sac56-d acl5-1 in the L*er* background was crossed to *acl5-1* in the Col-0 background. DNA was extracted from these F2 plants and used as a template for PCR-based mapping with SSLP and CAPS markers (Bell and Ecker, 1994; Konieczny and Ausubel, 1993). These markers were derived from the TAIR (http://www.arabidopsis.org). The primers used are shown in Table 2.

Genotypes of *acl5-1*, *sac52-d*, *sac53-d*, and *sac56-d* mutations were confirmed by the dCAPS analysis (Neff et al., 1998). Confirmation of homozygosity of the Salk insertion lines, *rack1a-1* and *rack1a-2* were achieved by PCR with their gene-specific primers and a T-DNA-specific primer. Primer sequences and restriction enzymes used for genotyping are listed in Table 3.

DNA sequences were analyzed with an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Plasmid construction and plant transformation

For a recapitulation of the *sac56-d* phenotype, the 2.9kb genomic fragment containing 893 bp upstream from the *SAC56* start codon and 376 bp downstream from the *SAC56* stop codon was amplified from *sac56-d acl5-1* genomic DNA with the primers, 56R-F, 5'-TTGCT CAGAT TATGG TCCGA-3', and 56R-R, 5'-GACAT TTGAA TTCGG TTTGA GCTTC-3'. PCR products

were subcloned into the pGEM-T easy vector (Promega), digested with *Cla*I and *Eco*RI, and ligated into similarly digested pBI101 (Clontech).

For analysis of GUS expression, the 5'-UTR of the *SAC51* genomic fragment was amplified from wild-type DNA by PCR using the primers, SAC5'-F, 5'-AGATC TAAGT GACCA ACGAA CA-3', and SAC5'-R, 5'-AGATC TAGAG GCATG CTCTT AG-3'. PCR products were digested with *Bgl*II, and ligated into similarly digested pBI101.

Transformation of *Arabidopsis thaliana* was performed as described in Harrison et al. (2006).

GUS assay

For histochemical staining of GUS activity, 1-week-old seedlings were prefixed for 20 min in ice-cold 90% (v/v) acetone, and were incubated in the GUS staining buffer (50 mM $NaPO_4$, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 0.1% Triton-X100, 1 mM X-Gluc, pH7.2) at 37°C for overnight. Seedlings were then treated with 70% ethanol to remove chlorophyll. For fluorometric assay, 10-day-old seedlings were ground with mortar and pestle in the GUS extraction buffer (50 mM sodium phosphate pH7.0, 10 mM EDTA, 0.1% Triton-X100, 0.1% *N*-lauroyl sarcosine (SLS), 10 mM ß-mercaptoethanol), and centrifuged. 10 µL of the supernatant was mixed with 190 µL of the GUS reaction buffer (1 mM 4-MUG in the GUS extraction buffer). The reaction mixture was incubated for 1 hr at 37°C, and the reaction was stopped with 2 mL of 0.2 M Na_2CO_3. The fluorescence was measured with an RF-1500 Spectrofluorophotometer (Shimadzu) at $\lambda_{355}/\lambda_{460}$. The protein content was measured using the Bradford method (Nacalai tesque).

RNA extraction and RT-PCR

Total RNAs from 10-day-old seedlings were extracted by the SDS-phenol method (Hanzawa et al., 1997). cDNAs were synthesized from 3 µg of total RNA using PrimeScript reverse transcriptase (Takara). The real-time PCR was performed with the DNA Engine Opticon2 System (Bio-Rad) using a KAPA SYBR FAST Universal qPCR Kit (Kapa Biosystems). *ACT8* and *UBQ10* were used as an internal control. All primers used in RT-PCR are listed in Table 1.

Histology and Microscopy

Cleared seedlings were prepared by a previously reported method (Yoshimoto et al., 2012). These samples were observed under a light microscope equipped with Nomarski DIC optics (DM5000B, Leica). GUS-stained seedlings were observed under a stereoscopic microscope.

CHAPTER I

Structure of the polyamines that functionally substitute for thermospermine

INTRODUCTION

Polyamines are small cationic molecules with two or more primary amino groups present in all living cells and play important roles in cell growth and development (Tabor et al,. 1984; Pegg,. 1986). Among the three major polyamines (putrescine, spermidine, and spermine), spermidine and spermine are formed successively from putrescine through the addition of an aminopropyl moiety of decarboxylated *S*-adenosyl methionine (dcSAM) by each specific aminopropyl transferase (APT), spermidine synthase (SPDS) and spermine synthase, respectively. Spermidine is a substrate for the hypusine modification of the eukaryotic translation initiation factor 5A (eIF5A), which is essential in all eukaryotic cells (Chattopadhyay et al., 2008). Due to their cationic nature, polyamines, in particular spermine, have high binding affinity to RNA, DNA, proteins, and other acidic substances, and participate in many cellular processes. One of the major functions of intracellular polyamines may be in regulating mRNA translation because most polyamines exist in polyamine–RNA complex within cells (Igarashi and Kashiwagi, 2010). Polyamines are also proposed to be a source of hydrogen peroxide, which is produced through polyamine oxidative degradation, and play a role in stress responses. In higher plants, polyamines are accumulated extracellularly in response to pathogen attacks and their degradation by apoplastic polyamine oxidase (PAO) results in the production of hydrogen peroxide, which may, in turn, activate defense pathways against pathogens (Kusano et al., 2008; Moschou et al., 2008).

Plant cells also contain thermospermine, a structural isomer of spermine, which was first detected in an extreme thermophile, *Thermus thermophiles* (Oshima, 1979). Thermospermine is formed from spermidine by the action of thermospermine synthase, an enzyme similar to spermine synthase (Knott et al., 2007). Phylogenetic analyses, however, suggest that thermospermine synthase was acquired by an algal ancestor of plants through horizontal gene transfer from archaea while spermine synthase evolved from SPDS in respective lineages of plants, animals, fungi, and bacteria (Minguet et al., 2008). Although thermospermine has been sporadically found in animal systems (Hamana et al., 2004), its function remains unknown. A previous study has shown that the *acaulis5* (*acl5*) mutant of *Arabidopsis thaliana*, which exhibits severe dwarfism with increased vein thickness and vascularization in stems (Hanzawa et al., 1997, 2000), is defective in the synthesis of thermospermine and exogenously-applied thermospermine partially restores the mutant phenotype (Kakehi et al., 2008). A study of the *thickvein* (*tkv*) mutant, an allele of *ACL5*, suggests that the boundary between veins and non-vein regions is defined by *ACL5/TKV* whose expression is specific to provascular cells (Clay and Nelson, 2005). Isolation and characterization of *suppressor of acl5* (*sac*) mutants, which more or less suppress the dwarf phenotype of *acl5*, suggest that thermospermine may have a role in the upstream open reading frame (uORF)-mediated translational control of a subset of genes including *SAC51*, a gene encoding a potential negative regulator of vascular differentiation (Imai et al., 2006,

2008). However, the precise mode of action of thermospermine remains to be understood. Because the *Arabidopsis spms* mutant, which is defective in spermine synthase, is wild-type in appearance (Imai et al., 2004), the structural difference between thermospermine and spermine must be critical for their respective functions. I therefore addressed structural features of polyamines that are required for the control of plant vascular differentiation and stem elongation. Here I show that norspermine, which has the C3C3 arrangement of carbon chains in common with thermospermine (Fig. 3), can functionally replace thermospermine.

RESULTS

Norspermine partially rescues the stem elongation defect of *acl5*

To compare the effect of norspermine with that of thermospermine and spermine, I used *acl5-1 spms-1* double mutants, which produce neither thermospermine nor spermine. My experiments revealed that daily application of norspermine and thermospermine but not of spermine to shoot tips of *acl5-1 spms-1* seedlings clearly rescued the stem growth defect (Fig. 4). I confirmed that, under the same growth condition, application of these polyamines to wild-type seedlings had no effect on the appearance of the adult flowering plants (data not shown).

Norspermine and thermospermine down-regulate *ACL5* and *SAMDC4/BUD2*

To know the effect of norspermine at the molecular level, transcript levels of genes involved in the synthesis and catabolism of polyamines were investigated. Norspermine, thermospermine, or spermine was added to liquid cultures of *acl5-1 spms-1* seedlings. Quantitative reverse transcription-PCR experiments revealed that the level of the *acl5-1* transcript in *acl5-1 spms-1*, which is up-regulated by a probable feedback response to thermospermine deficiency (Hanzawa et al., 2000), was drastically reduced by norspermine as well as by thermospermine (Fig. 5A). The *ACL5* expression in the wild-type was also reduced by norspermine and thermospermine but not by spermine. Because *spms-1* represents a T-DNA insertion allele in the 5′ leader sequence of *SPMS* and contains no detectable transcripts for *SPMS*, I examined the transcript level of *SPMS* in wild-type seedlings and confirmed that *SPMS* expression was neither responsive to norspermine, spermine, nor thermospermine treatment (Fig. 5B). The *Arabidopsis* genome contains four genes encoding SAM decarboxylase, *SAMDC1* to *SAMDC4/BUD2* (Ge et al., 2006). I found that, among the four genes, only *SAMDC4/BUD2* showed much higher expression in *acl5-1 spms-1* mutants than in the wild-type and this was attributed to the effect of the *acl5-1* allele (data not shown). *SAMDC4/BUD2* was significantly down-regulated by norspermine and thermospermine (Fig 5C). Transcript levels of *SPDS1* and *SPDS2*, two genes encoding SPDS, are not affected in *acl5-1 spms-1* (Imai et al., 2004). These genes were not responsive to exogenous spermine, thermospermine, and norspermine in *acl5-1 spms-1* (Fig. 5D).

Norspermine and thermospermine affect the expression of key genes for vascular development

I next examined expression of the genes involved in the regulation of vascular differentiation. ATHB8 and PHABULOSA (PHB) are members of the class III homeodomain-leucine zipper

(HD-ZIP III) proteins which are known to be essential for vascular development (Baima et al., 2001; Prigge et al., 2005). Transcripts of these genes are expressed at much higher levels in *acl5-1* compared to the wild-type (Kakehi et al., 2008; Imai et al., 2006). My experiments revealed that *ATHB8* and *PHB* were also down-regulated by both norspermine and thermospermine in *acl5-1 spms-1* (Fig. 5E).

A previous study identified a bHLH-type transcription factor SAC51 as a probable negative regulator of vascular differentiation because the suppression of the *acl5-1* phenotype by a dominant suppressor allele, *sac51-d*, is attributed to overexpression of *SAC51* (Imai et al., 2006). The *SAC51* transcript level is up-regulated by thermospermine (Kakehi et al., 2008). The *Arabidopsis* genome contains three additional genes that are closely related to *SAC51*; *At5g09460*, *At5g50010*, and *At1g29950*. Hereafter I name them *SACL1, SACL2,* and *SACL3*, respectively. *acl5-1 spms-1* mutants showed normal levels of these transcripts (data not shown). I found that transcript levels of *SAC51* and *SACL1* were up-regulated by both thermospermine and norspermine while those of *SACL2* and *SACL3* were not altered by these polyamines (Fig. 5F).

Norspermidine cannot substitute for thermospermine in regulating stem elongation

The C3C3 arrangement of carbon chains present in thermospermine and norspermine is also contained in a triamine, norspermidine (Fig. 3). Unlike thermospermine and norspermine, however, norspermidine did not rescue the dwarf phenotype of *acl5-1* by its daily application to the shoot apex. I also examined expression of *SAC51* in *acl5-1 spms-1* mutants by using the *GUS* reporter gene fused to the promoter and the 5′ leader sequence of *SAC51* (Imai et al., 2006). GUS staining is detected in most tissues but preferentially in vascular tissues in wild-type seedlings (Imai et al., 2006), and only weak staining was detected in *acl5-1 spms-1* seedlings (Fig. 6A). External norspermidine, spermidine, and spermine at 0.1 mM had no inductive effect on the GUS activity, while thermospermine and norspermine at 0.1 mM increased it drastically in *acl5-1 spms-1* seedlings (Fig. 6B). Because the efficacy of triamines to influence biological events may be generally lower than that of tetramines, I examined the effect of norspermidine at higher concentrations (0.2-2 mM) but observed no influence on the GUS activity (Fig. 6C). I further examined the effect of homocaldopentamine (C3C3C3C4, Fig. 3) on the GUS activity but no effect was detected at 0.1 mM (Fig. 6B).

Xylem differentiation is blocked by norspermine and thermospermine

Microscopic observation of the *acl5-1 spms-1* seedlings grown in the liquid MS medium supplemented with a polyamine showed that thermospermine and norspermine but not spermine resulted in an apparent reduction in the development of lignified vessel elements, which is observed as brown-colored tissues (Hirakawa et al., 2008) in Fig. 7, suggesting inhibitory effects of these polyamines on xylem vessel differentiation.

Tetramines containing the C3C3 structure can substitute for thermospermine

I further examined the effect of artificially synthesized tetramines containing the C3C3 structure, aminoethyl norspermidine (C3C3C2) and aminopentyl norspermidine (C3C3C5) (Fig. 3). *acl5-1 spms-1* seedlings carrying the SAC51-GUS construct were grown in the presence of 0.1 mM aminoethyl norspermidine or aminopentyl norspermidine. They showed

reduction in the development of lignified xylems (Fig. 7). In addition, aminoethyl norspermidine and aminopentyl norspermidine increased the GUS activity in *acl5-1 spms-1* seedlings carrying the SAC51-GUS construct while homocaldopentamine had no effect (Fig. 8A). I also confirmed that expression of *ACL5* was reduced in response to aminoethyl norspermidine, and aminopentyl norspermidine (Fig. 8B).

DISCUSSION

Norspermidine and norspermine have been detected in a few plant species including alfalfa and cotton, and are predicted to be synthesized successively by each specific APT or a single APT with broad substrate specificity from 1,3-diaminopropane, which is produced by degradation of spermidine or spermine by PAO (Kuehn et al., 1990). However, because there are no putative genes for such APT identified in the *Arabidopsis* whole genome sequence, it is unlikely that *Arabidopsis* tissues contain these uncommon polyamines. With the use of *Arabidopsis* mutants deficient in the synthesis of spermine and thermospermine, I demonstrated that tetramine containing C3C3 arrangement, i.e. norspermine, can function as a substitute for thermospermine in the promotion of stem elongation, the repression of lignified vessel differentiation, and the regulation of a subset of genes. The *Arabidopsis spms* mutant has been shown to be more sensitive to high salt and drought conditions than the wild-type (Yamaguchi et al., 2007) but my preliminary experiments revealed that the *acl5-1 spms-1* mutant seedlings grown in the presence of norspermine showed no obvious increase in the tolerance to these stresses, suggesting that norspermine may not substitute for spermine. On the other hand, norspermidine and homocaldopentamine did not substitute for thermospermine in my experiments. These results suggest that, in addition to the C3C3 structure of carbon chains, four amino moieties are important for the action of thermospermine and norspermine. It is possible, however, that the different effects of each exogenous polyamine are due to different uptake efficiencies. Polyamine transport systems remain to be elucidated in plants.

This study revealed that expression of one of four *SAMDC* genes, *SAMDC4/BUD2*, was increased in *acl5-1* mutants and down-regulated by thermospermine and norspermine. This is reminiscent of the *ACL5* expression, which is under negative feedback control by thermospermine (Kakehi et al., 2008). Because the *bud2* mutant shows bushy and dwarf phenotype (Ge et al., 2006), it is possible that the supply of dcSAM for the synthesis of thermospermine is mediated predominantly by *SAMDC4/BUD2* and that the *bud2* mutant lacks thermospermine, resulting in the dwarfism. *SAMDC1* has two uORFs and its translation is down-regulated by excess spermidine and spermine through the uORF-mediated pathway (Hanfrey et al., 2005). *SAMDC2* and *SAMDC3* also have the two conserved uORFs but *SAMDC4/BUD2* does not (Franceschetti et al., 2001). Detailed studies of the *bud2* mutant and *BUD2* expression profiles are needed to clarify the relation between *ACL5* and *SAMDC4/BUD2*.

The “uncommon” polyamines such as thermospermine, norspermine, and other longer or branched polyamines, were initially detected in thermophilic bacteria and have been implicated in nucleic acid stabilization and mRNA translation under extreme growth conditions (Uzawa et al., 1993; Oshima, 2007). Previous studies suggested that thermospermine plays a role in overcoming the inhibitory effect of the uORFs of the *SAC51* transcript on the main ORF translation, although the precise mode of action is as yet to be elucidated (Imai et al., 2006, 2008). If so, it is likely that the observed increase in the *SAC51*

transcript level by thermospermine and norspermine represents translation-dependent stabilization of existing transcripts rather than new transcription. A peptide sequence similar to that encoded by the longest uORF of *SAC51* is conserved within that of *SACL1*, *SACL2*, and *SACL3*, but *SACL2* and *SACL3* showed no clear increase in their transcript levels in response to thermospermine and norspermine. Thus, independent of the conserved peptide sequences encoded by these uORFs, thermospermine and norspermine might act on specific RNA sequences and enhance the main ORF translation. Because *ACL5* is highly expressed in provascular cells (Clay and Nelson, 2005), thermospermine may be responsible for specifying tissues that express a subset of genes including *SAC51* and *SACL1*. Further identification of the target genes whose transcript level is affected by thermospermine and norspermine will help to elucidate the action mechanism of these tetramine. Moreover, intracellular polyamines mainly interact with RNA molecules (Igarashi and Kashiwagi, 2000), and it is possible that the C3C3 structure in tetramines plays important roles in an interaction between itself and the *SAC51* mRNA and controls the translation efficiency of *SAC51* main ORF. Finally, I should also note the possibility that the phenomena described above are due to versatile actions of exogenous polyamines and specific modification or oxidation of thermospermine and norspermine play a role in the observed effects.

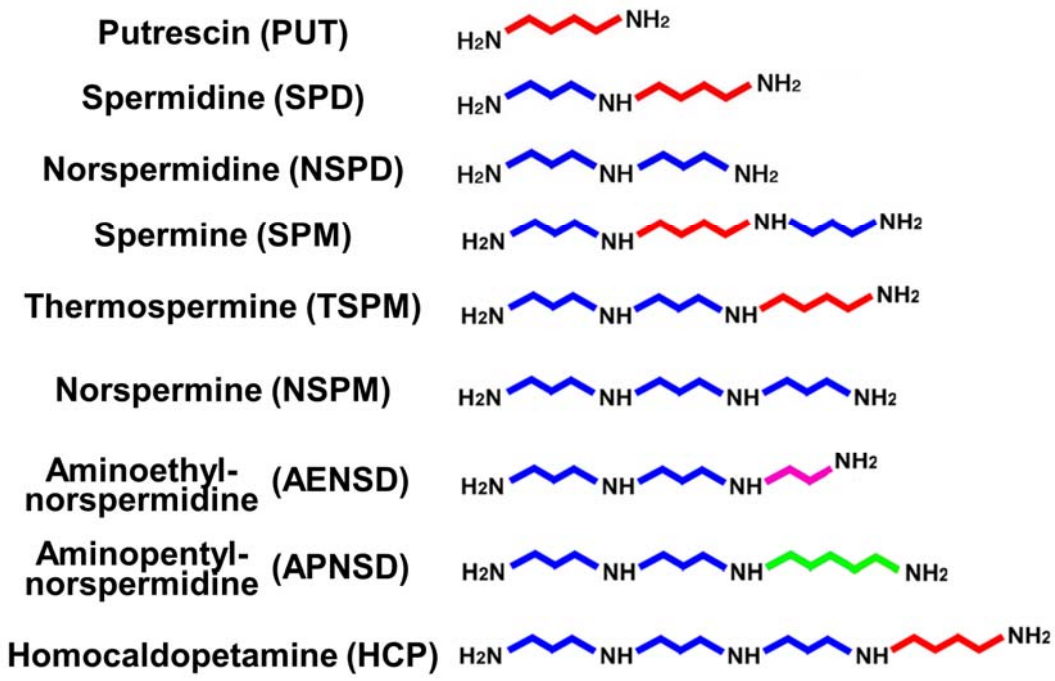

Fig.3

Molecular structures of the three major polyamines and polyamines used in this study.
Purple, blue, red, and light green lines indicate carbon chains of C2, C3, C4, and C5, respectively.

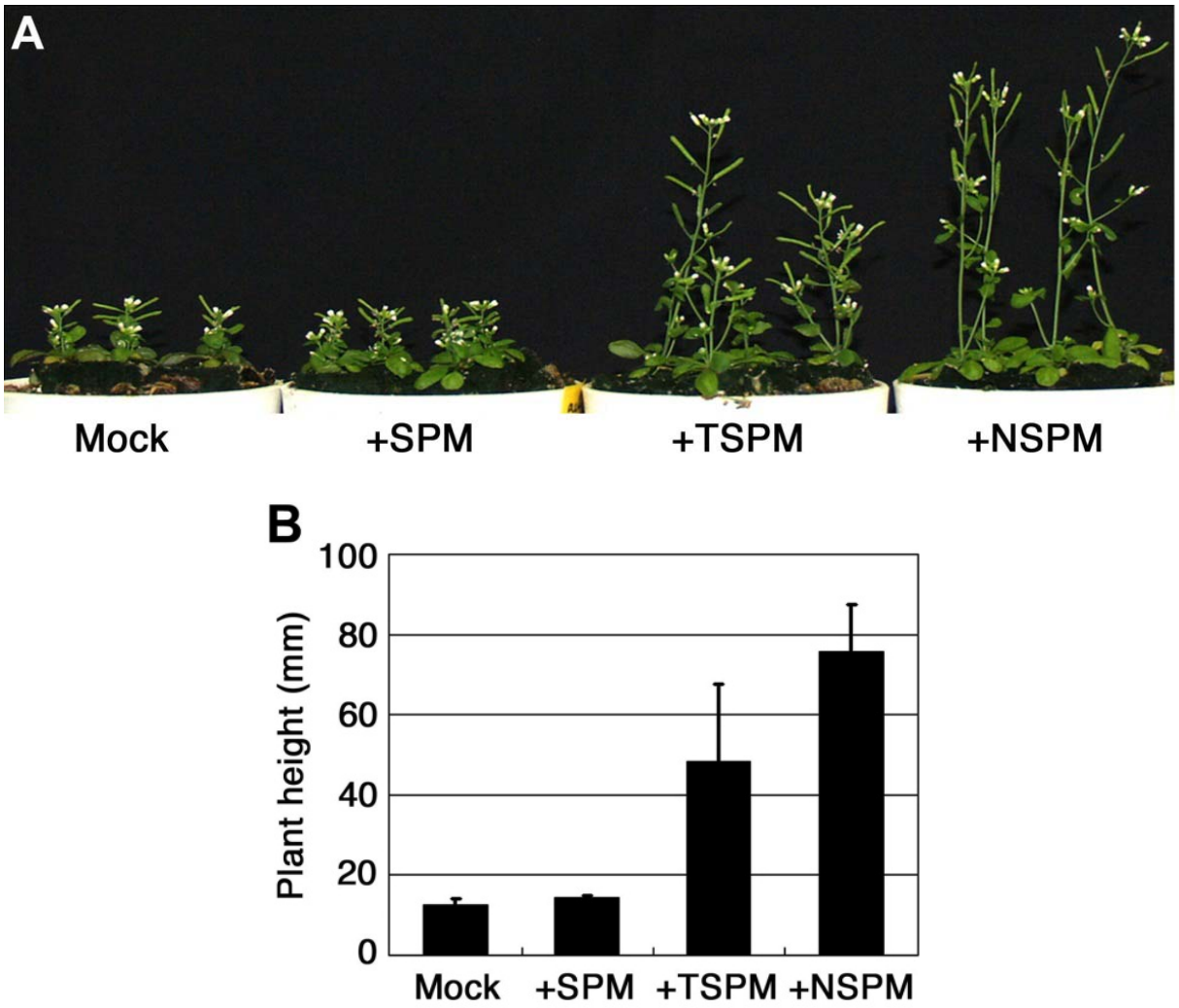

Fig. 4

Effects of exogenous application of polyamines on the growth of *acl5-1 spms-1* mutants. (A) Phenotypes of 40-day-old *acl5-1 spms-1* plants. Spermine (SPM), thermospermine (TSPM), or norspermine (NSPM) was applied to the shoot tip of the plants everyday from 10 days after germination as 40 µl of the 0.1 mM solution. (B) Plant height of 40-day-old *acl5-1 spms-1* plants treated as in (A). Error bars represent S.D. values of three independent experiments.

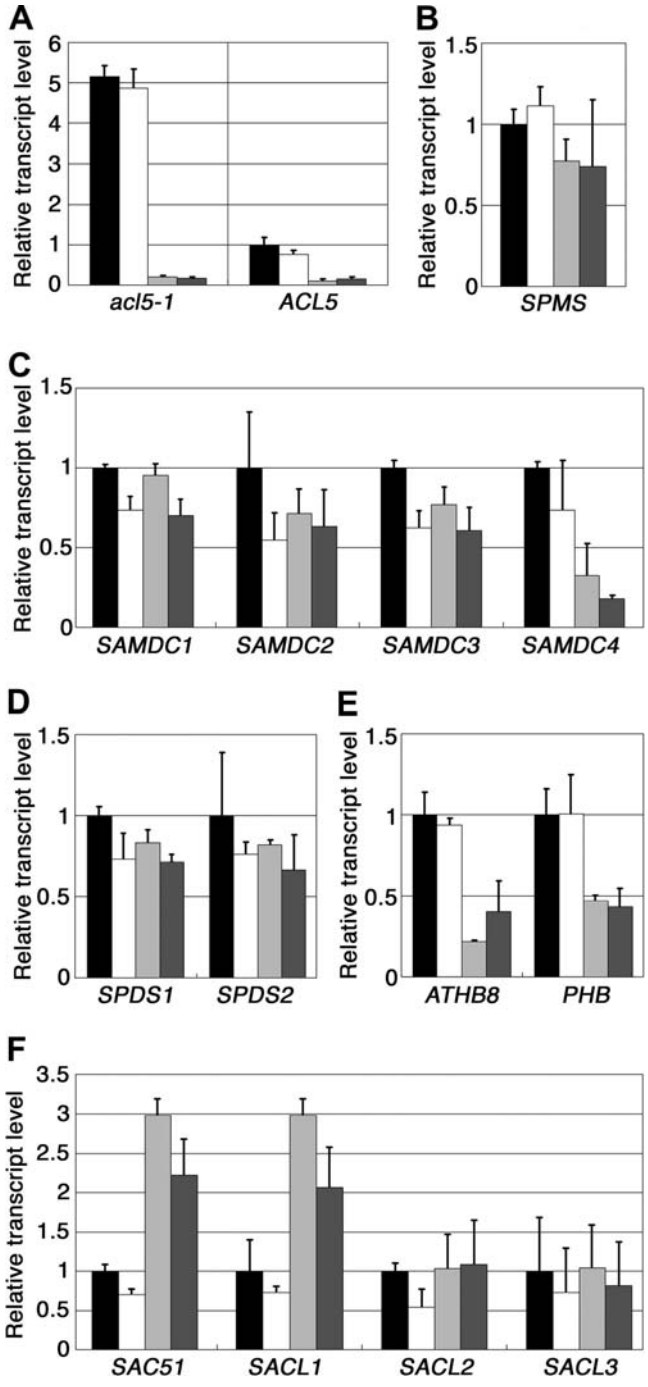

Fig. 5

Effects of exogenous application of polyamines on gene expression.

Relative transcript levels of *ACL5* (A), *SPMS* (B), *SAMDC* genes (C), *SPDS* genes (D), HD-ZIP III genes (E), and *SAC51*-like genes (F) were examined by quantitative RT-PCR. Wild-type (the right panel of A and B) and *acl5-1 spms-1* (the left panel of A, C, D, E, and F) seedlings were grown for 10 days in liquid MS medium and treated with mock (black bars), spermine (white bars), thermospermine (light gray bars), and norspermine (dark gray bars) at 0.1 mM for 24 h, respectively. Transcript levels were set to 1 in mock-treated controls. *acl5-1* in the left panel of (A) represents the mutant transcript derived from the *acl5-1* allele. Transcript levels were normalized to *ACT8* transcript. Error bars represent S.D. values of three independent experiments.

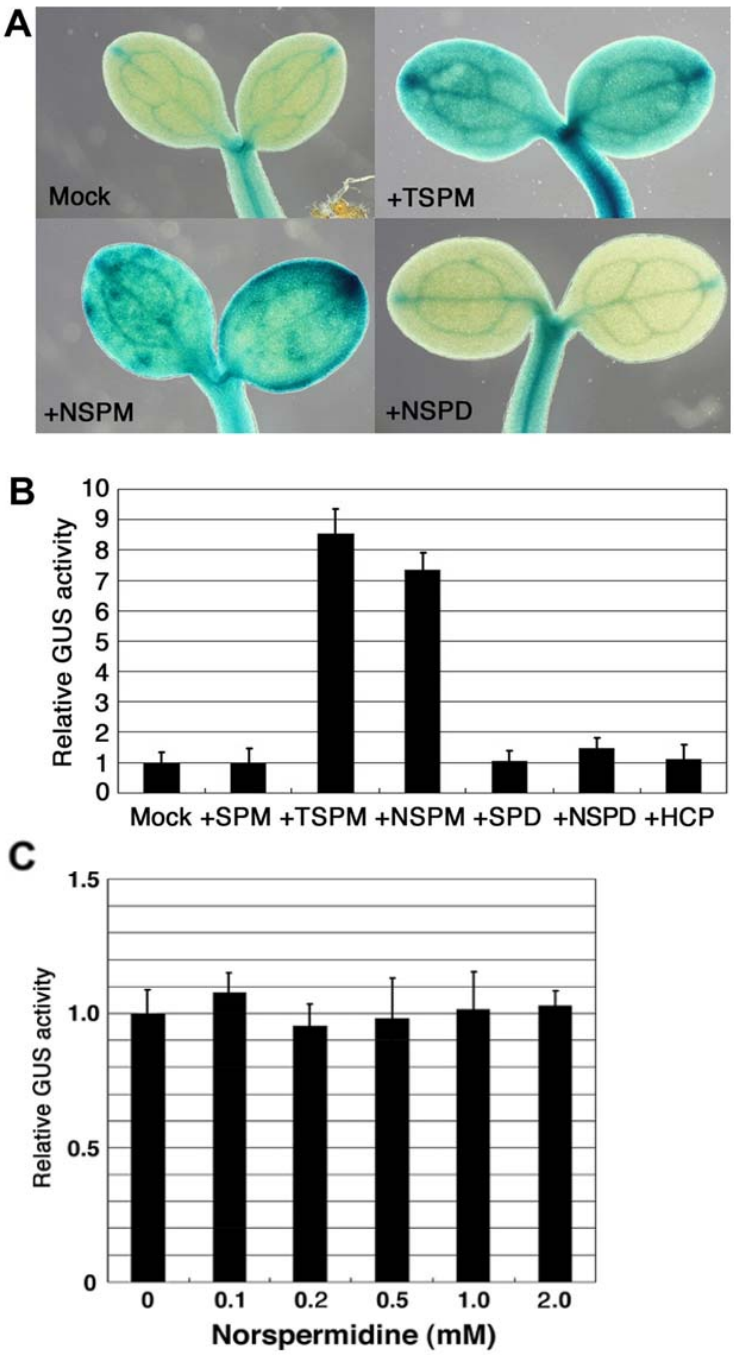

Fig. 6

Effects of exogenous application of polyamines on the *SAC51-GUS* fusion gene expression. *acl5-1 spms-1* seedlings carrying the *GUS* reporter gene fused with the *SAC51* promoter and its 5′ leader region were grown for 3 days in MS solutions, treated with each polyamine at 0.1 mM for 24 h, and stained (A) or assayed (B) for GUS activity. (C) GUS activity in *acl5-1 spms-1* seedlings treated with norspermidine at 0.1 to 2.0 mM for 24 h. Error bars represent S.D. values of three independent experiments.

SPM, spermine; TSPM, thermospermine; NSPM, norspermine; SPD, spermidine; NSPD, norspermidine; HCP, homocaldopentamine.

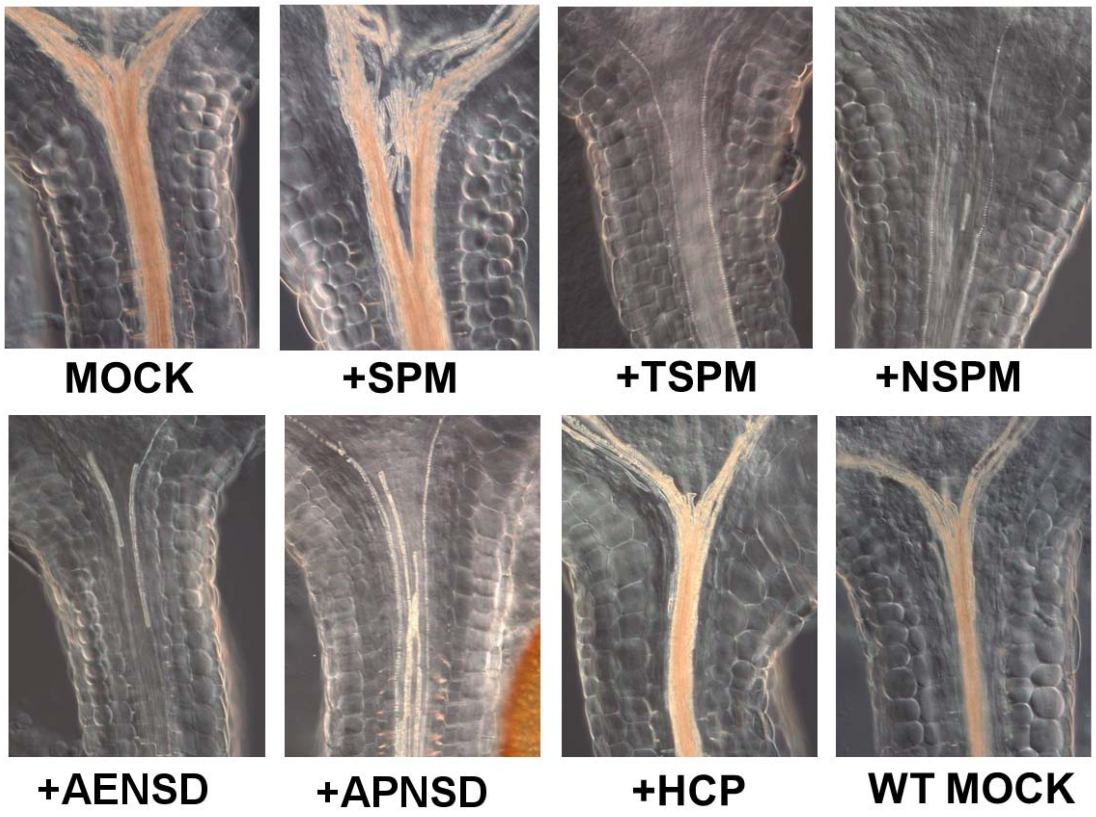

Fig. 7
Effects of polyamines in the growth medium on xylem vessel differentiation in *acl5-1 spms-1*. Seedlings were grown for 7 days in the liquid MS medium supplied with each polyamine at 10 μM. Hypocotyls were observed under light microscopy.
AENSD, aminoethyl nosrpermidine; APNSD, aminopentyl norspermidine.

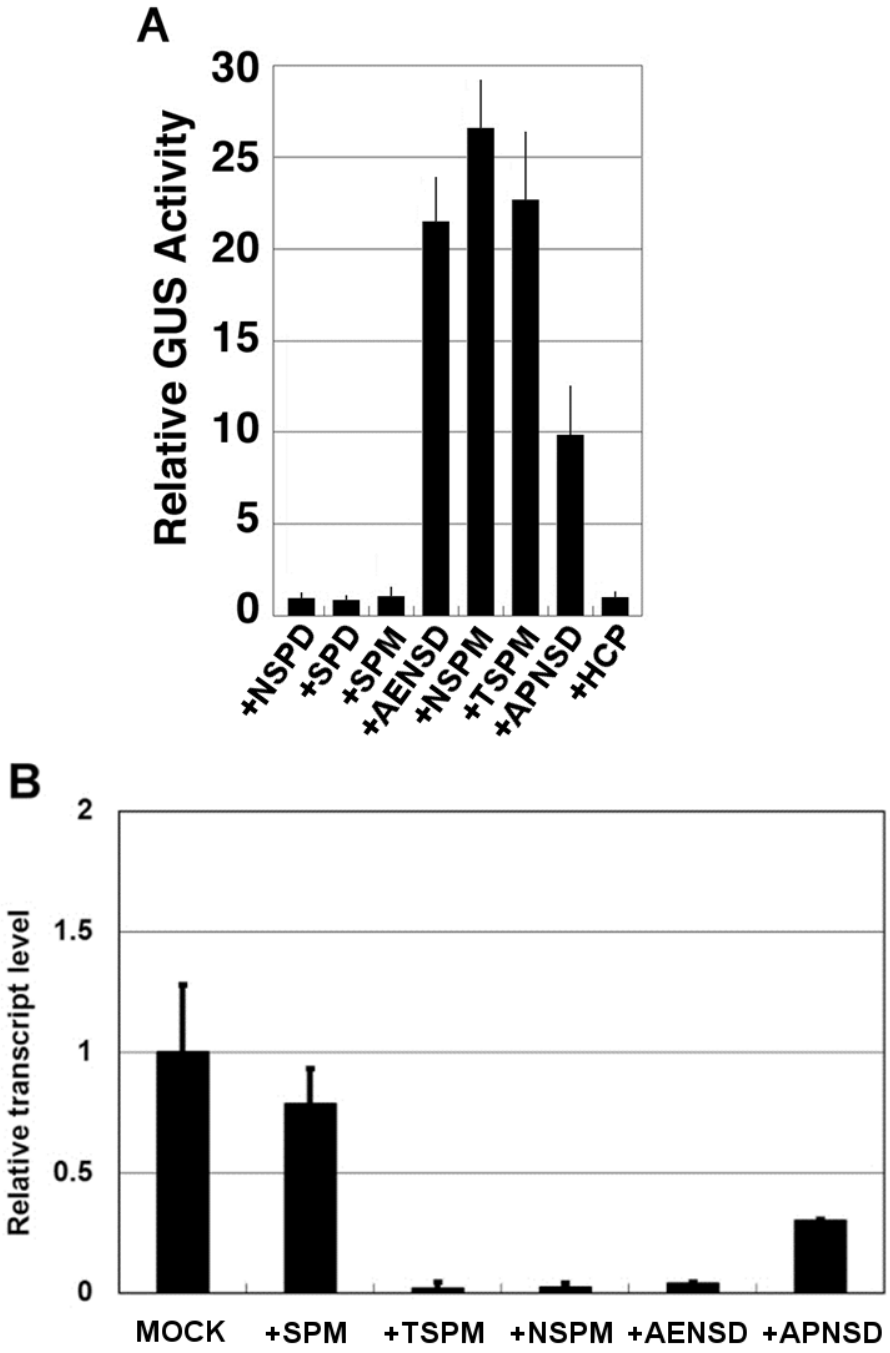

Fig. 8

Effects of exogenous application of various polyamines on *SAC51* and *ACL5* in *acl5-1 spms-1*. (A) The GUS activity in *acl5-1 spms-1* seedlings carrying the *SAC51-GUS* construct consisting of the *SAC51* promoter, its 5' leader sequence containing uORFs, and the *GUS* reporter gene. (B) The *ACL5* transcript level in *acl5-1 spms-1*. Plants were grown for 7 days in liquid MS medium supplemented with each polyamine at 0.1 mM. Transcript levels were normalized to *ACT8* transcript. Error bars represent S.D. values of three independent experiments.

Table 1. Primers for real time RT-PCR.

Gene name		Sequence
ACT8	F	5'-GTGAGCCAGATCTTCATCGTC-3'
	R	5'-TCTCTTGCTCGTAGTCGACAG-3'
UBQ10	F	5'-CACACTCCACTTGGTCTTGCGT-3'
	R	5'-AAGATCAACCTCTGCTGGTCCG-3'
ACL5	F	5'-ACCGTTAACAGCGATGCTTT-3'
	R	5'-CCGTTAACTCTCTCTTTGATTCTTCGATCC-3'
SPMS	F	5'-ACATATCCAAGCGGCGTGAT-3'
	R	5'-CCTCTTCAAGAGTTCTACAAAG-3'
SPDS1	F	5'-ACCTCTCACAAACCCTAGTT-3'
	R	5'-TCCATATGTTGCAGACTGGA-3'
SPDS2	F	5'-AATCTCTTACTCACTGTCTC-3'
	R	5'-TCCATATGTTGCAGACTGGA-3'
SAC51	F	5'-AATTGCCAGGCTGAGTACTT-3'
	R	5'-GACCGACCTACTATATCCTT-3'
SACL1	F	5'-GATGGATAATTGTCAGGCTG-3'
	R	5'-CAGTTTAAGCCAAGGGAACT-3'
SACL2	F	5'-GGGGTCGTCTTTTTTCTTCA-3'
	R	5'-TAGAAAACATGTCGGTGGTG-3'
SACL3	F	5'-TTGGAAGAACCAAAGATGCC-3'
	R	5'-TTCAGCCTGGCAAACTTGAA-3'
SAMDC1	F	5'-GGTTTCTGGAAACAAGGAGT-3'
	R	5'-TCTTGGAGCAGTTTGAGTAG-3'
SAMDC2	F	5'-TCCTCTACATAAAAAGCGTG-3'
	R	5'-GGAACCAGAGCTTTGAGAAA-3'
SAMDC3	F	5'-ATTGCTCTGTTCTTCACAGG-3'
	R	5'-CAACCACAGTTAGTAAGTGG-3'
SAMDC4	F	5'- ATGGCAGTGTCTGGGTTCGA -3'
	R	5'- CTATTTCCGACGAGGCGTGA -3'
ATHB8	F	5'-AGCGTTTCAGCTAGCTTTTGAG-3'
	R	5'-CAGTTGAGGAACATGAAGCAGA-3'
PHB	F	5'-TGCTATAGAAAGGAGTCCT-3'
	R	5'-ATTGCCCTCTGGCGTTTTCT-3'

CHAPTER II

Effects of the mutations of ribosomal components and thermospermine on the *SAC51* mRNA stability

INTRODUCTION

The *acaulis5* (*acl5*) mutant of *Arabidopsis thaliana* shows a dwarf phenotype due to reduced cell length in stem internodes (Hanzawa et al., 1997). *ACL5* encodes a thermospermine synthase (Kakehi et al., 2008). Thermospermine is a structural isomer of spermine and was first identified from thermophilic bacteria, *Thermus thermophilus* (Oshima 1979). A recent study suggests that thermospermine is widely distributed in the plant kingdom (Takano et al., 2012). To elucidate the molecular mode of action of thermospermine in stem elongation, suppressor mutants of *acl5* that lead to a restoration of the phenotype have been isolated and named *sac* (Imai et al., 2006). The *sac51-d* mutant is a dominant allele of *SAC51* and completely suppresses the dwarf phenotype of *acl5* without thermospermine. The 5′ leader of *SAC51* mRNA contains five upstream open reading frames (uORFs) preceding the initiation codon of the main ORF, which encodes a bHLH transcription factor, and the *sac51-d* allele contains a premature stop codon in the 4th uORF (Imai et al., 2006). This uORF is conserved in higher plant genomes (Hayden and Jorgensen, 2007). Such conserved uORFs may play a role in the translational regulation of the main ORF. For instance, a uORF of *AtbZIP11* mRNA is involved in sucrose-dependent translation of the main ORF, which encodes a transcription factor (Rahmani et al., 2009). Two uORFs of the *AdoMetDC/SAMDC1* mRNA encoding an *S*-adenosyl methionine decarboxylase are known to be required for polyamine-dependent translation repression of the *S*-adenosyl methionine decarboxylase (Ivanov et al, 2010). Given the fact that intracellular polyamines are mainly associated with RNA molecules (Igarashi and Kashiwagi, 2000), it is possible that thermospermine interacts with the 5' leader sequence of *SAC51* and plays an important role in the translation enhancement of the *SAC51* main ORF. The *SAC51* mRNA is suggested to be a target of nonsense-mediated mRNA decay (NMD), because mRNA levels of *SAC51* family genes are increased in the mutant of *UPF1*, which is essential factor of NMD (Yoine et al., 2006). NMD is one of RNA surveillance mechanisms that detects a premature stop codon located upstream of the last exon-exon junction of the mRNA and triggers its degradation (Baker and Parker, 2004). The relationship between translational efficiency and mRNA stability of the *SAC51* mRNA remains to be investigated.

The second *sac* mutant, *sac52-d* also has a dominant effect over the wild-type *SAC52* gene. *SAC52* encodes a ribosomal protein L10A, RPL10A (Imai et al., 2008). RPL10 is a component of the 60S large ribosomal subunit, and a highly conserved protein in eukaryotes (Dick and Trumpower, 1998). In yeast, RPL10 is a multifunctional translational regulator, operating in 60S subunit biogenesis, export, and joining with the 40S subunit (Pachler et al., 2006). The *sac52-d* allele in the background of *acl5-1* has been shown to promote translation of the *SAC51* main ORF (Imai et al., 2008). On the other hand, the *sac53-d* mutant represents a semi-dominant allele and its responsible gene encodes the Receptor for Activated C Kinase 1, so-called RACK1 (Kawano, unpublished). RACK1 has been also shown to be a component of the ribosome (Link et al., 1999). In *Arabidopsis*, some mutants of ribosomal proteins show

multiple developmental phenotypes (Byrne, 2009). Mutations of ribosomal protein genes, *PIGGYBACK* (*PGY*), *ASSYMETRIC LEAVES ENHANCER* (*AE*), and *RPL4D*, enhance the phenotype of *asymmetric leaves1* (*as1*) or *as2* mutants and affect leaf polarity (Pinon et al., 2008; Szakonyi et al., 2011; Yao et al., 2008; Horiguchi et al., 2011). A mutant of *short valve1* (*stv1*) whose responsible gene encodes *RPL24B* enhances the phenotype of *ettin/arf3* (*ett*) mutants. *ETT* and the related *AUXIN RESPONSE FACTOR5/MONOPTEROS* (*ARF5/MP*) mRNAs also have uORFs. In the absence of these uORFs, translation efficiency of the main ORF is increased. These results suggest that the interaction between ribosomal proteins and uORFs plays a role in regulating the translation efficiency of these mRNA (Nishimura et al., 2005).

In this study, I confirmed that *SAC53* encodes RACK1A and further found that the gene responsible for *sac56-d* encodes RPL4A. Moreover, my experiments revealed that thermospermine stabilizes the *SAC51* mRNA, and that mutations of ribosomal proteins, *sac52-d*, *sac53-d*, and *sac56-d*, also have stabilizing effects on the *SAC51* mRNA.

RESULTS

There are more than 10 *sac* mutants isolated so far. I focused my study on three ribosome mutants, *sac52*, *sac53*, and *sac56*. As shown in Fig. 9, *sac52-d acl5-1* and *sac56-d acl5-1* plants are normal in appearance, whereas *sac53-d acl5-1* shows 67% recovery of the wild-type height. *acl5-1* plants heterozygous for *sac52-d* are also wild-type in appearance, whereas those heterozygous for *sac53-d* or *sac56-d* show less recovery of the height than homozygous mutants, indicating that *sac53-d* and *sac56-d* are semi-dominant. *sac53-d* and *sac56-d* single mutants are indistinguishable from wild-type plants (data not shown).

SAC53 encodes RACK1A

In a previous study, *sac53-d* was found to contain a single base substitution in *RACK1A* (Kawano, unpublished). The *RACK1A* gene has one intron and encodes a protein of 327 amino acids. In *sac53-d*, a G-to-A substitution results in a premature stop codon (W261X) in the last WD40 domain (Fig. 10A). The *Arabidopsis* genome contains three *RACK1* homologues (Chen et al., 2006). W261 is conserved in all *Arabidopsis* and human RACK1 isoforms but not in yeast (Fig. 10B). However, it remained to be proved whether the suppressor phenotype in *sac53-d* is indeed caused by a mutation in *RACK1A* or not. Thus, I examined whether the phenotype of *acl5-1* can be suppressed by other alleles of *RACK1A* or not. I crossed *acl5-1* to *rack1a-1* and *rack1a-2* (Fig. 10A, Chen et al., 2006), and obtained *acl5-1 rack1a-1* and *acl5-1 rack1a-2* double mutants. As shown in Fig. 11, these mutants also partially restored the stem growth.

SAC56 encodes a ribosomal protein L4A

sac56-d has been identified from additional screening of an ethyl methanesulfonate (EMS)-mutagenized population of *acl5-1* for suppressor mutants (Kawano, unpublished). The *SAC56* locus was mapped on the upper arm of chromosome 3. Fine mapping placed the *SAC56* locus to about 130-kb region between the marker, F11F8-1 and F8A24-1 (Figure 12A). This 130-kb region contains 49 genes. Sequencing of these genes from the *sac56-d acl5-1* revealed a G-to-A point mutation in *At3g09630*. *At3g09630* encodes a ribosomal protein L4A (RPL4A,

Fig. 12B). The G-to-A base substitution in *sac56-d* changes Gly75 to Arg75 (Fig. 12C). RPL4 is a highly conserved ribosomal protein of the large subunit among all organisms. The *Arabidopsis* genome has two active genes, *RPL4A* and *RPL4D*, and two pseudogenes, *RPL4B* and *PRL4C*, (Barakat et al., 2001). These two genes show high similarity (95.0%). Gly75 is conserved in eukaryotes and prokaryotes (Figure 12C).

To confirm that this mutation is really responsible for suppression of the *acl5-1* phenotype, I transformed *acl5-1* plants with a genomic copy of the *SAC56R* gene fragment derived from the *sac56-d* allele and tested whether it can restore the phenotype or not. As shown in Fig. 11, all three transgenic lines obtained showed recovery of plant height.

To determine the relationship between *SAC56* and *SAC52* or *SAC53*, I crossed these mutants to each other and generated *sac* trans-heterozygotes. The results revealed that all of *sac52-d*/+ *sac53-d*/+ *acl5-1*, *sac52-d*/+ *sac56-d*/+ *acl5-1* and *sac53-d*/+ *sac56-d*/+ *acl5-1* plants showed an additive phenotype on stem elongation (Fig. 9B).

sac mutations compensate for thermospermine deficiency in *acl5-1*

I examined the effect of three *sac* mutations in ribosomal components on gene expression in *acl5-1*. Previous studies revealed that the genes for thermospermine synthesis, *ACL5* and *SAMDC4/BUD2*, and those involved in vascular development, *ATHB8* and *VND7*, are up-regulated in *acl5-1*, and down-regulated by exogenous thermospermine (Kakehi et al., 2008; Imai et al., 2006; Yoshimoto et al., 2012). My experiments revealed that the transcripts of these genes were present at normal levels in *acl5-1 sac52-d*, *acl5-1 sac53-d*, and *acl5-1 sac56-d* (Figure 13). Excess xylem formation in *acl5-1* cotyledons is enhanced by treatment with 2,4-D-IOE (Yoshimoto et al., 2012). 2,4-D-IOE is the isooctyl ester of a synthetic auxin, 2,4-D. I examined the effect of 2,4-D-IOE on xylem development in *sac* mutants. My results revealed that all of *sac52-d*, *sac53-d*, and *sac56-d* suppressed the effect of 2,4-D-IOE in *acl5-1* and showed normal development of veins in cotyledons (Figure 14).

I further examined whether *sac52-d*, *sac53-d*, and *sac56-d* suppress the dwarf phenotype of *bud2-2* or not. The gene for *bud2-2* is *SAMDC4*, one of four genes encoding an *S*-adenosyl methionine decarboxylase in *Arabidopsis*. The mild dwarf phenotype of *bud2-2*, which has been reported by Ge et al. (2006), suggests that the phenotype is attributed to thermospermine deficiency. I crossed each *sac* mutant to *bud2-2* and found that *bud2-2 sac52-d* and *bud2-2 sac56-d* plants restored growth to wild-type levels while *bud2-2 sac53-d* showed moderate recovery (Fig. 15).

SAC51-GUS expression in *sac53-d* and *sac56-d*

A previous study suggested that uORF-mediated translational control of *SAC51* is essentially involved in shoot growth and xylem differentiation (Imai et al., 2006). The *sac52-d* mutation of RPL10A also appears to enhance translation of *SAC51* (Imai et al., 2008). To determine whether *sac53-d* and *sac56-d* mutations affect the translation efficiency of *SAC51*, I examined GUS expression in transgenic *sac53-d* and *sac56-d* lines that express the *GUS* reporter gene under the control of the Cauliflower mosaic virus (CaMV) 35S promoter and the *SAC51* 5' leader sequence containing five uORFs. The 35S-*SAC51* 5'-GUS fusion construct was introduced into the wild-type genome and then transferred to *acl5-1*, *sac53-d acl5-1*, and *sac56-d acl5-1* plants by crosses. Transgenic lines homozygous for the *GUS* gene were used for further experiments. GUS activity was about 5-fold higher in wild-type than in *acl5-1*. The

GUS activity was increased also in *sac53-d acl5-1* and *sac56-d acl5-1* compared with *acl5-1* single mutants (Figure 16B). On the other hand, the GUS staining pattern in *sac53-d acl5-1* and *sac56-d acl5-1* was similar to that in the wild-type (Figure 16A).

SAC51 mRNA is stabilized by *sac* mutations

There are three genes with high similarity to *SAC51* in the *Arabidopsis* genome, *SACL1*, *SACL2*, and *SACL3* (Imai et al., 2006). The mRNA levels of these genes are increased in the *low-beta-amylase1* (*lba1*) mutant (Yoine et al., 2006). Since the gene responsible for *lba1* encodes a UPF1 RNA helicase, which is involved in NMD, *SAC51* and *SACL* mRNAs may be targets of NMD. I confirmed that the *SAC51* mRNA level was increased in *lba1/upf1* and also in *upf3*, which represents a mutant of another factor involved in NMD (Hori et al., 2005) compared with that in the wild-type (Fig. 17A). Time-course assays of mRNA stability using cordycepin revealed that the *SAC51* mRNA was more destabilized in *acl5-1* than in the wild-type but was stabilized by exogenous treatment with thermospermine (Fig. 17B and C). Finally, the *SAC51* mRNA stability was examined in *sac52-d acl5-1*, *sac53-d acl5-1* and *sac56-d acl5-1*. These results revealed that the *SAC51* mRNA was stabilized by these *sac* mutations, although the effect of *sac53-d* on the *SAC51* mRNA stability was lower than that of *sac52-d* and *sac56-d* (Fig. 17C).

DISCUSSION

In this study, I found that, in addition to *sac52-d*, *sac53-d* and *sac56-d* mutations up-regulated the GUS activity under the control of the *SAC51* uORFs. These *sac* mutations in ribosomal components had additive effects on the recovery of stem growth (Fig. 9), suggesting that these ribosomal components participate in different aspects of translational regulation.

Effects of *sac53-d* on *SAC51* expression

My results revealed that different alleles of *RACK1A* partially suppressed the dwarf phenotype of *acl5*, confirming that *SAC53* encodes RACK1A. RACK1A is a core component of the 40S ribosomal subunit (Link et al., 1999). In yeasts, however, the interaction between ribosome and RACK1 is not essential for survive (Dresios et al., 2006). In mammals, RACK1 is located in the region near the mRNA exit tunnel and directly interacts with rRNA (Sengupta et al., 2004). RACK1 has also been shown to act as a scaffold protein to recruit multiple signal molecules (i.e. PKC, eIF6, and Integrin-B), and mediate various signal transduction pathways (Nilsson et al., 2004; Adams et al., 2011). The *Arabidopsis* genome has three homologs of *RACK1* (*RACK1A*, *B* and *C*). T-DNA insertion mutants of these genes have various effects on physiological and developmental events, and the triple mutant is embryonic lethal (Chen et al., 2006; Guo et al., 2008). Since *rack1a* mutants show severer defects in growth compared to *rack1b* and *rack1c, RACK1A* may be a major factor of RACK1 (Guo et al., 2008). RACK1 is a beta-propeller protein comprising seven WD40 repeats (Wall et al., 1995; Ullah et al., 2008; Adams et al., 2011). *sac53-d* has a nonsense mutation in the 6th WD40 domain (Figure 2B). This domain mediates interaction between ribosome and various proteins in yeasts and animals (Nilsson et al., 2004). In yeasts, RACK1 induces nascent peptide-dependent translation arrest and triggers the no-go-mediated mRNA decay (NGD) pathway (Kuroha et al., 2010). NGD is one of mRNA surveillance mechanisms in eukaryotic

cells. This degradation system is triggered when ribosomes are stalled by a stem-loop containing mRNA thereby Dom34 and Hbs1 are involved in initial recognition of stalled ribosomes (Doma and Parker, 2006; Passos et al., 2009). These factors are conserved in *Arabidopsis* and rice (Atkinson et al., 2008), although it remains unclear whether or not the NGD system functions in plants. It will be interesting to examine whether mutants of these factors also suppress the *acl5* phenotype or not. It is possible that the *sac53-d* mutation uncouples the *SAC51* mRNA from the decay and consequently increases translational efficiency of its main ORF.

sac56-d is a semi-dominant allele of *RPL4A*

Ribosomal protein L4 (RPL4) is a component of the 60S large ribosomal subunit and is highly conserved among eukaryotes (Dresios et al., 2006). The *Arabidopsis RPL4* family is composed of two full-length genes, *RPL4A* and *RPL4D*, and two pseudogenes, *RPL4B* and *RPL4C* (Barakat et al., 2001). Previous studies indicated that mutations in ribosomal protein genes enhance *as1*/*as2* leaf dorsoventral polarity defects (Pinon et al., 2008; Yao et al., 2008; Szakonyi et al., 2011), and that the *rpl4d* mutations have similar effects (Horiguchi et al., 2011). *RPL4A* and *RPL4D* are functionally redundant, and loss-of-function mutations in these genes result in various phenotypes, including narrow pointed first leaves, an abnormal cotyledon number, and short roots and hypocotyls (Rosado et al., 2010). An analysis of the crystal structure of the ribosome from *E. coli* suggests that RPL4 and RPL22 (RPL17 in eukaryotes) are located near the constricted region of the nascent peptide exit tunnel of the ribosome (Nissen et al., 2000). RPL4 and RPL22 contain elongated "tentacles" that reach into the peptide exit tunnel, and this domain is not essential for translation elongation (Zengel et al., 2003). In bacteria, a number of regulatory peptide sequences, such as SecM, TnaC, and Crb^{CmlA}, stall in ribosomes and cause translational arrest to regulate the expression of downstream genes (Collier et al., 2004; Gong et al., 2002; Stokes et al., 1991; Cruz-Vera et al., 2011). This translation arrest is released by mutations of tentacle domains of RPL4 and RPL22, and these data suggest that these tentacle have an important role in the nascent peptide-dependent ribosome stalling (Nakatogawa et al., 2002; Lawrence et al., 2008). In eukaryotes, it is well known that ribosome stalling is caused by the arginine attenuator peptide (AAP), which is encoded by a uORF of *Arg-2*, and it is suggested that a nascent peptide of AAP interact with RPL4 and RPL17 during translation in the ribosome (Wu et al., 2012). Moreover, mutations in the tentacles of RPL4 and RPL22 result in resistance to antibiotics such as erythromycin and chloramphenicol (Wittmann et al., 1973; Zaman et al., 2007). *rpl4* mutants of *Arabidopsis* also exhibit resistance to erythromycin and chloramphenicol (Rosado et al., 2010) suggesting that functions of the constriction site in the ribosomal tunnel are conserved between eukaryotes and prokaryotes.

A structural comparison of eukaryotic and prokaryotic RPL4 proteins suggests that the amino acid altered in *sac56-d* is located in the tentacle domain of RPL4. My results revealed that *sas56-d* enhanced translation of the *SAC51* main ORF (Fig. 16), suggesting that the *sac56-d* mutation affects conformation of the exit tunnel and that the translation of the main ORF of *SAC51* is enhanced by this structural change. This is also consistent with the semi-dominant trait of *sac56-d*. T-DNA insertion mutations of *RPL4D* and *RPL5A* result in a decreased translation of auxin responsive factors (ARFs) containing uORFs (Rosado et al., 2012). Taken together, it is most likely that RPL4 plays a role in the uORF-mediated translational control of the gene expression.

Control of the *SAC51* mRNA stability by thermospermine and NMD

The *Arabidopsis* genome has three genes with high similarity to *SAC51*, *SACL1* (At5g09460), *SACL2* (At5g50010), and *SACL3* (At1g29950). It has been previously reported that the transcripts of these genes are accumulated in the *upf1* mutant (Yoine et al., 2006). UPF1 is an integral component of the nonsense-mediated mRNA decay (NMD) machinery. NMD is an mRNA surveillance pathway that degrades mRNA containing a premature termination codon (PTC). The core NMD machinery is comprised of the factors UPF1, UPF2, and UPF3, and they are all essential for NMD to recognize PTCs (Baker and Parker, 2004; Conti and Izaurralde, 2005; Chang et al., 2007). In mammalian cells, the induction of NMD requires the presence of an exon-junction complex downstream of a PTC and/or a long 3'-UTR (Chang et al., 2007; Hogg et al., 2010). Therefore, uORF-containing transcripts are predicted to be a major class of natural NMD targets (Baker and Parker, 2040). In tobacco, uORF-containing mRNAs have been shown to be degraded by the NMD pathway, and this result indicates that plant uORFs can also trigger NMD (Nyiko et al., 2009). My result that *SAC51* mRNA was highly accumulated in *upf1* and *upf3* (Fig. 17) consistently indicates that *SAC51* mRNA is also a target of NMD (Fig. 18).

I also found that *SAC51* mRNA is unstable without thermospermine but is stabilized by exogenous thermospermine or mutations of *sac52-d, sac53-d,* and *sac56-d*. In yeast and mammals, polyamines cause structural changes in stem-loops of mRNA, and stimulate the ribosome shunting (Nishimura et al., 2009; Uemura et al., 2009). Ribosome shunting is a process that allows the ribosome to physically bypass the 5'-UTR and to permit translation initiation at the correct start codon (Pooggin et al., 2000). Taken together, it is possible that thermospermine causes a conformational change in the stem-loop structure of the 5' region of *SAC51* mRNA, leads to the bypass of the mRNA surveillance, and consequently results in stabilization of the *SAC51* mRNA and enhancement of the main ORF translation (Fig. 18). Alternatively, thermospermine might promote leaky scanning of the uORF AUG. Leaky scanning is a mechanism that allows 40S small subunits to skip the first AUG and access to downstream AUG codons. In plants and mammals, expression of the gene for *S*-adenosyl methionine decarboxylase (AdoMetDC), an enzyme catalyzing the synthesis of polyamine substrate, involves translational control by leaky scanning (Ivanov et al., 2010).

On the other hand, stabilization of the *SAC51* mRNA by *sac52-d, sac53-d,* and *sac56-d* mutations of ribosomal components raises a possibility that these *sac* mutations cause the release of the stalled ribosome from the *SAC51* 4th uORF or destabilize ribosome-mRNA complex to enhance translation reinitiation from the main ORF. Another possibility is that these ribosomal mutations increase the frequency of leaky scanning of the *SAC51* 4th uORF (Fig. 18). Given that the *SAC51* mRNA is a target of NMD, the 4th uORF of *SAC51* whose termination codon locates upstream of a splicing site is likely a trigger of NMD because spliced exon junctions downstream of a termination codon can induce NMD. These possibilities may be consistent with dominant or semi-dominant phenotypes of *sac52-d, sac53-d,* and *sac56-d*. As described above, *sac53-d* representing a mutation in RACK1 might be defective in the NGD response. The result that *sac53-d* had a relatively weak effect on the *SAC51* mRNA stability compared to *sac52-d* and *sac56-d* might reflect the difference between the process involving RACK1 and that involving RPL10 and RPL4.

Finally, I generated *acl5 upf1* and *acl5 upf3* double mutants and examined whether *upf* mutations can suppress the *acl5* phenotype or not. But they did not suppress the dwarf phenotype (data not shown). NMD may be involved in many aspects of plant growth and

development and its defect may not only stabilize the *SAC51* mRNA but also have pleiotropic effects on the growth.

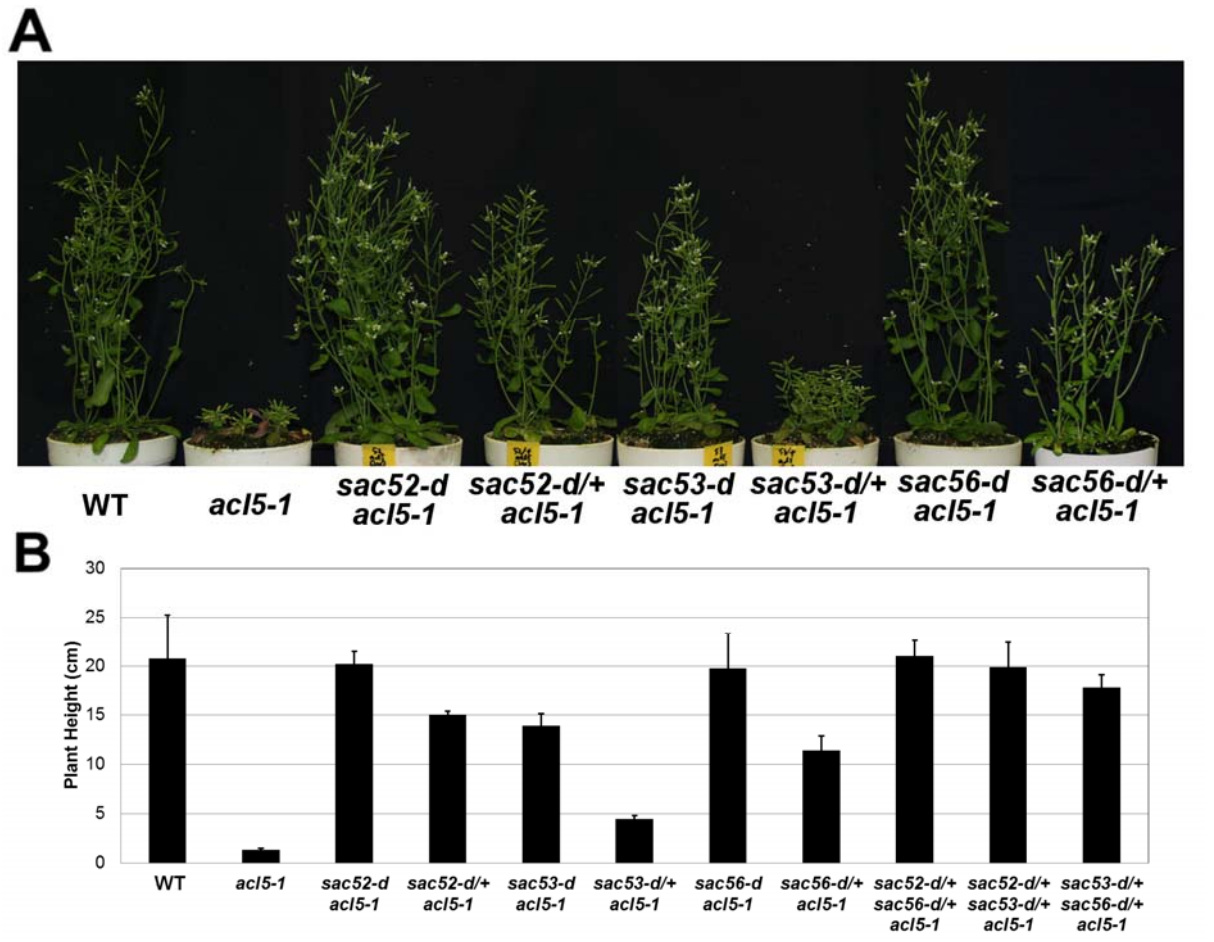

Fig. 9

Phenotypes of *acl5-1* and *sac* mutations.

Gross morphology (A) and plant height (B) comparison between wild-type, *acl5-1*, *sac* homozygous/heterozygous mutants in the *acl5-1* background, and *sac* trans-heterozygotes of 40-day-old plants. Error bars indicate S.D. values (n=6).

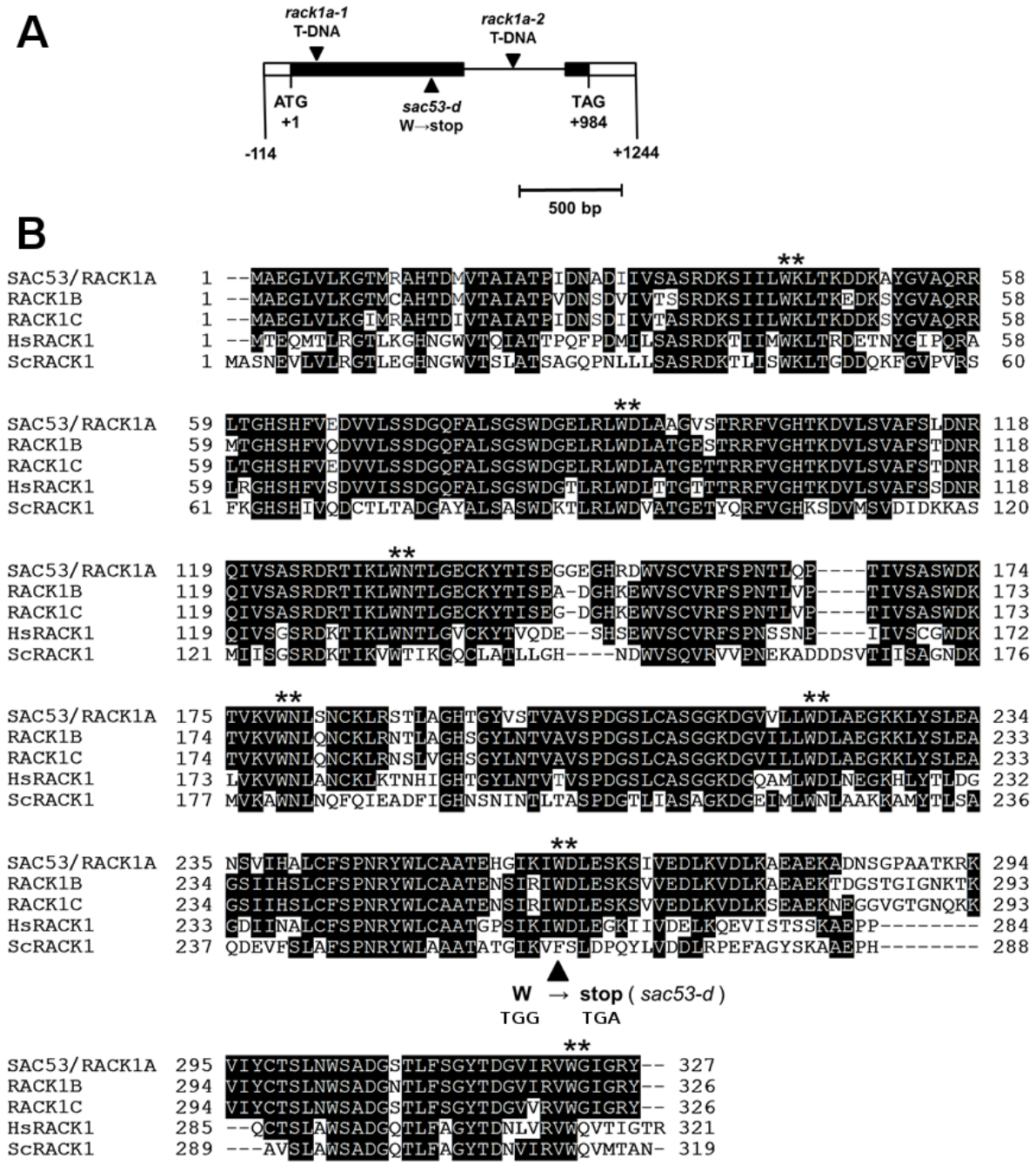

Fig. 10

Sequence alignment of the three *Arabidopsis* RACK1 proteins.

(A) Exon/intron structure of *SAC53/RACK1A*. The 5' and 3' UTRs, and the coding region are indicated by white and black boxes, respectively. Arrowheads indicate the positions of mutations. (B) Comparison of amino acid sequences of RACK1. *Arabidopsis* SAC53/RACK1A (accession number: NP_173248.1), RACK1B (accession number: AEE32329), and RACK1C (accession number: AEE76051) are aligned with *Homo sapiens* RACK1 (accession number: AAH32006) and *Saccharomyces cerevisiae* RACK1 (accession number: DAA10013) using Clustal W. Vertically-shaded boxes indicate conserved positions. Asterisks indicate the WD40 domain. The arrowhead indicates the position of *sac53-d* mutation.

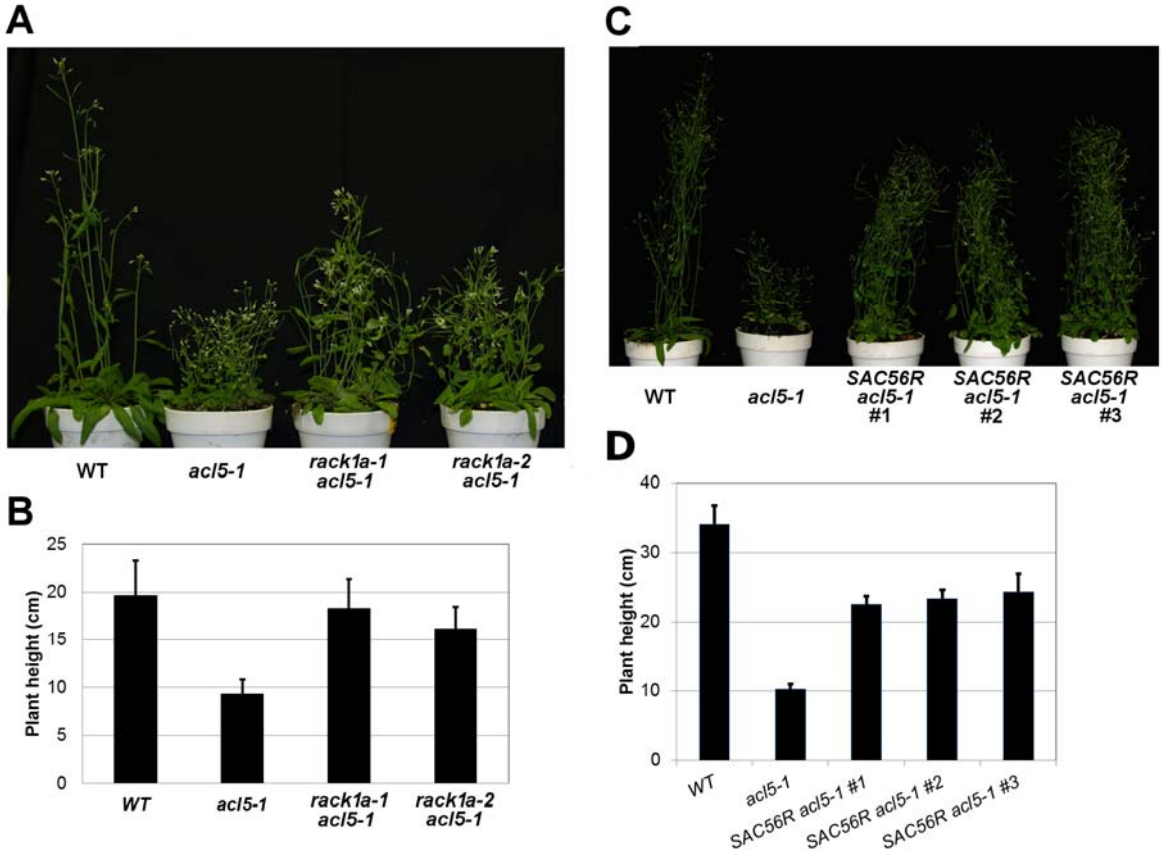

Fig. 11

Recapitulation of *sac53-d* and *sac56-d* by plant transformation.

(A, B) *rack1a-1* and *rack1a-2*, which represent two different T-DNA insertion alleles of *RACK1A*, suppressed the *acl5-1* mutant phenotype. (C, D) A genomic fragment of *sac56-d* could restore the phenotype of *acl5-1* plants. All three lines (#1-3) are homozygous for both *acl5-1* and the *SAC56R*. These lines restored the stem elongation. All plants are Col-0 background. Error bars indicate S.D. values (n=6).

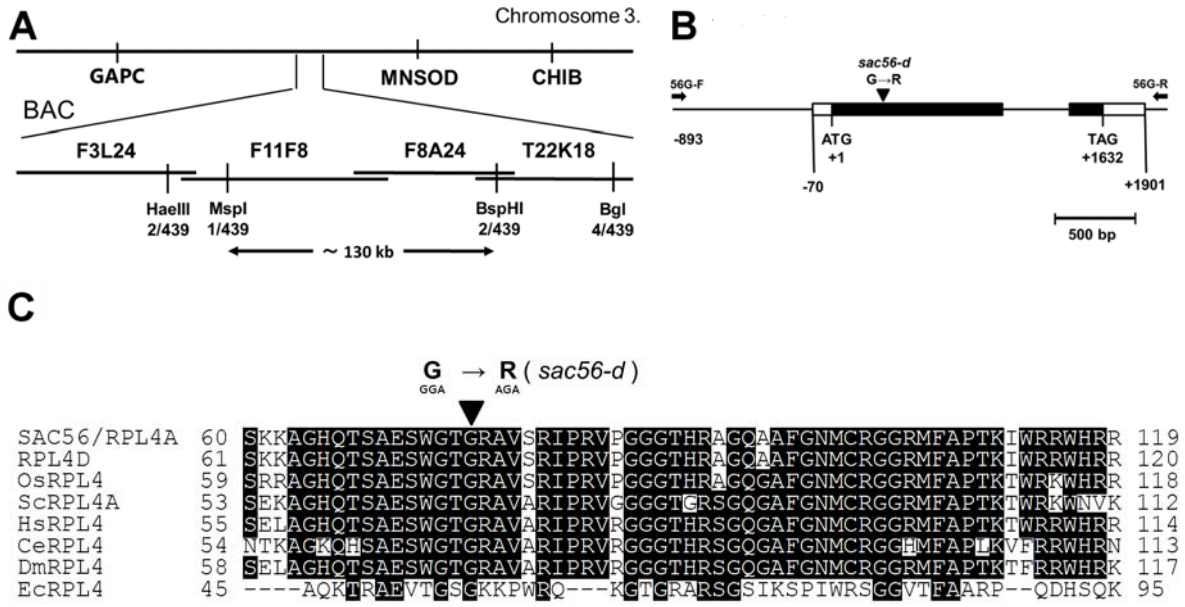

Fig. 12

Map-based cloning of *SAC56*.

(A) Map around the *SAC56* locus on chromosome 3. The positions of DNA markers are shown. The ratio indicates the number of recombinants per the total number of chromosomes. (B) Exon/intron structure of *SAC56/RPL4A*. The 5' and 3' UTRs, and the coding region are indicated by white and black boxes, respectively. The arrowhead indicates the position of *sac56-d* mutation. (C) Comparison of amino acid sequences of RPL4. *Arabidopsis* SAC56/RPL4 (accession number: AAP37854) and RPL4D (accession number: AED90529) are aligned with *Oryza sativa* RPL4 (accession number: NP_001059041), *Saccharomyces cerevisiae* RPL4A (accession number: NP_009587), *Homo sapiens* RPL4 (accession number: NP_000959), *Caenorhabditis elegans* RPL4 (accession number: CCD61249), *Drosophila melanogaster* RPL4 (accession number: AAG22173) and *Escherichia coli* RPL4 (accession number: ACI76839) using Clustal W. Vertically-shaded boxes indicate conserved positions. The arrowhead indicates the position of *sac56-d* mutation.

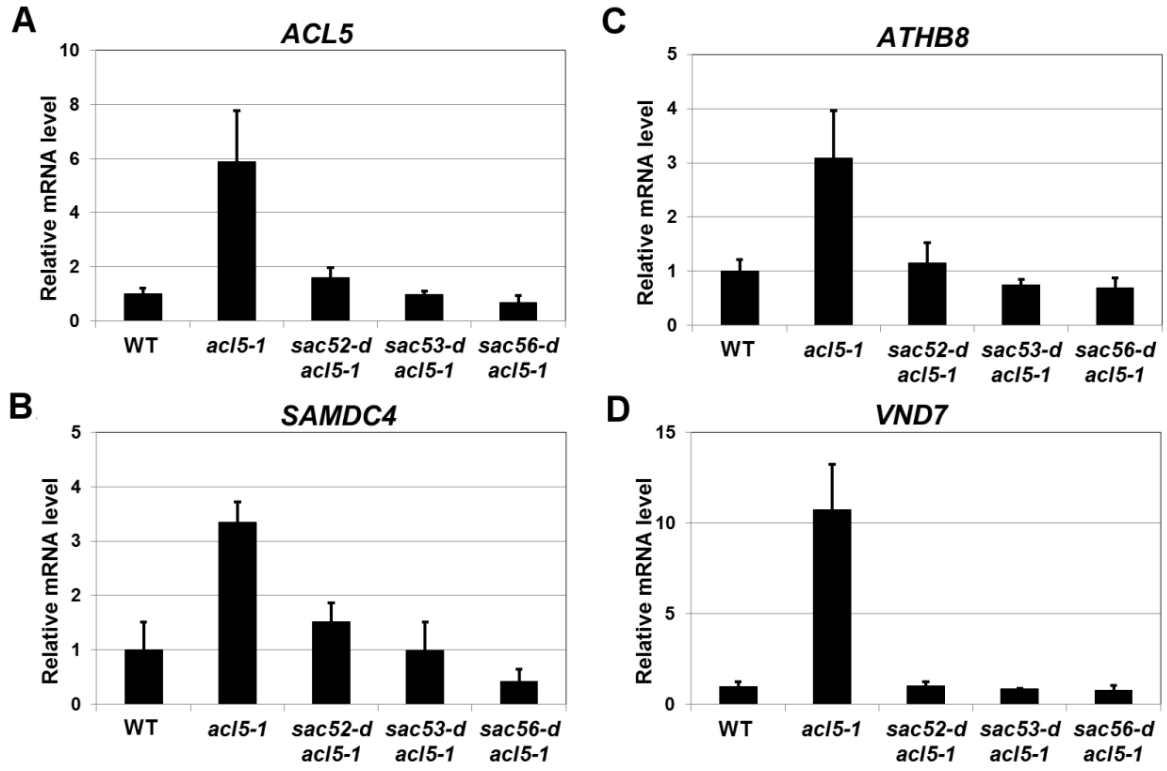

Fig. 13

Effects of *sac* mutations on gene expression.

Relative mRNA levels of *ACL5* (A), *SAMDC4* (B), *ATHB8* (C) and *VND7* (D) were examined by quantitative RT-PCR. Seedlings were grown for 10 days in liquid MS medium. Transcript levels were set to 1 in wild-type (WT). Transcript levels were normalized to *ACT8* transcript. Error bars represent S.D. values of three independent experiments.

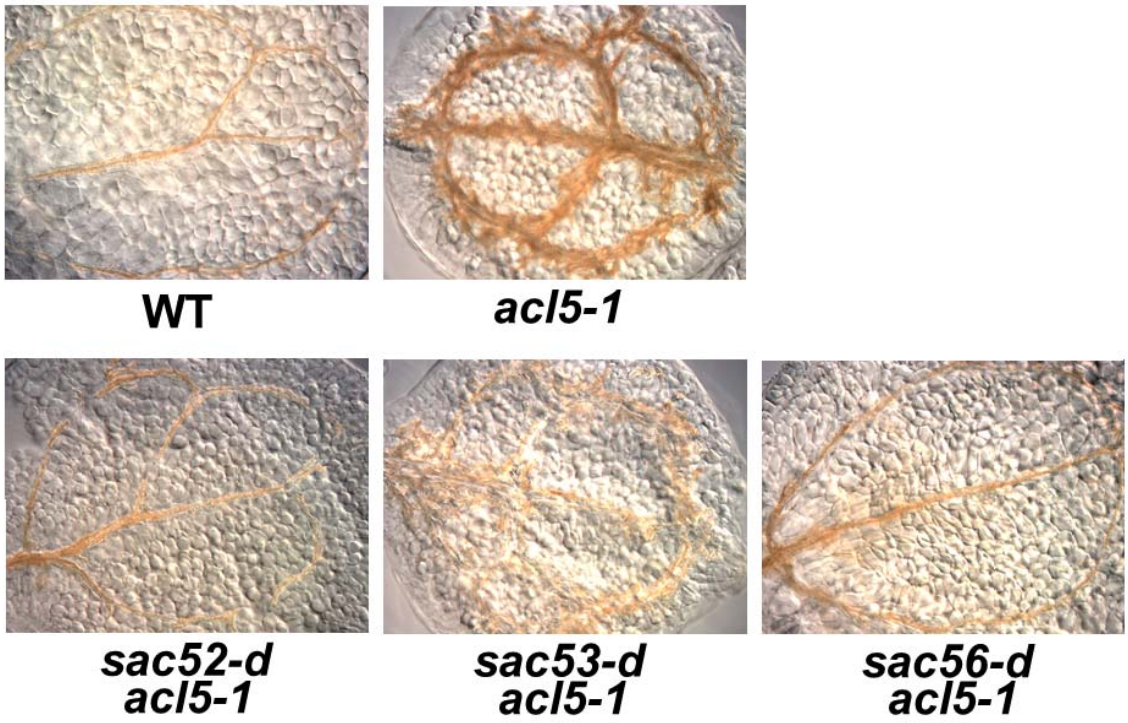

Fig. 14

Effects of exogenous treatment of 2,4-D-IOE.

Seedlings were grown for 7 days in the liquid MS medium constantly supplied with 2,4D-IOE at 10 μM. Cotyledons were observed under light microscopy.

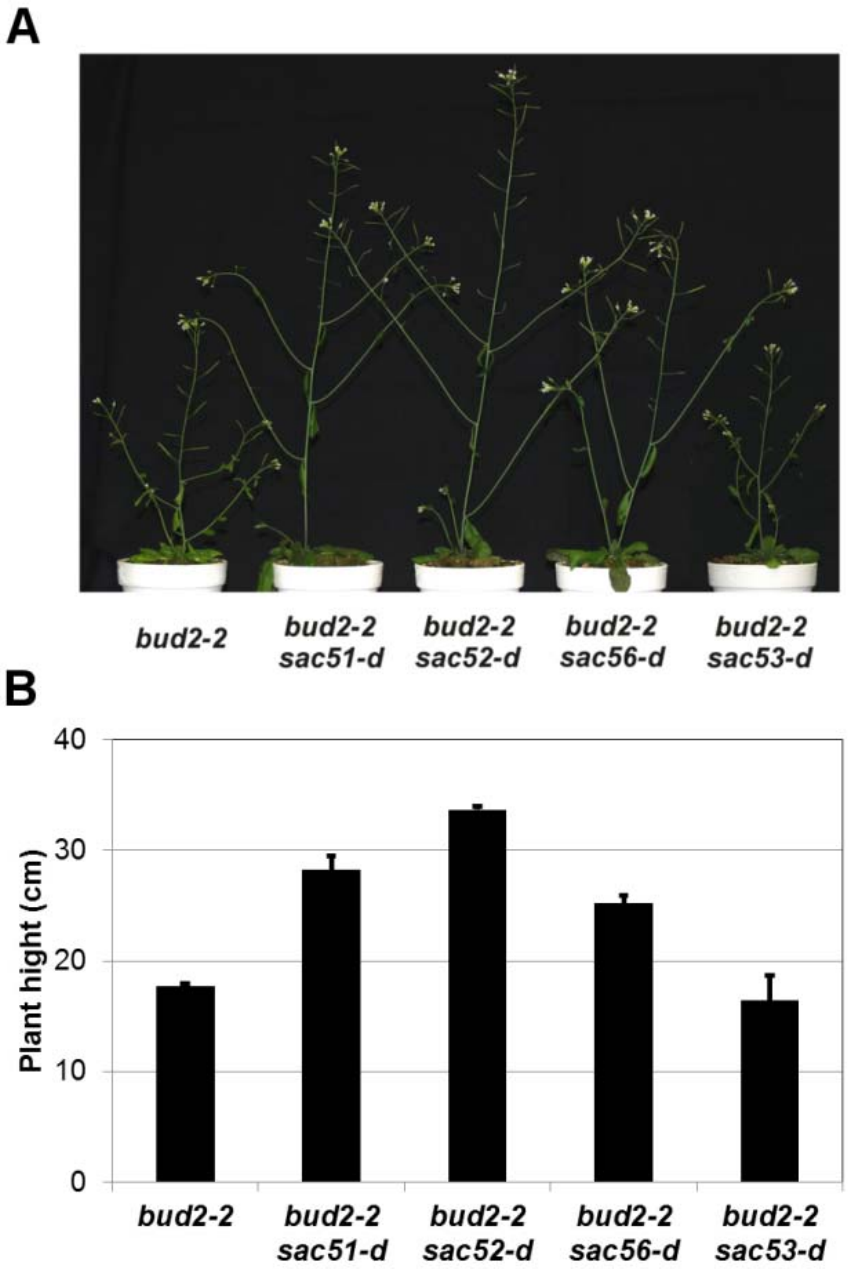

Fig. 15

bud2-2 mutant is suppressed by *sac51-d*, *sac52-d*, and *sac56-d* mutations.

Gross morphology (A) and plant height (B) comparison between *bud2-2 and bud2-2 sac* double mutants of 40-day-old plants. Error bars indicate S.D. values (n=6).

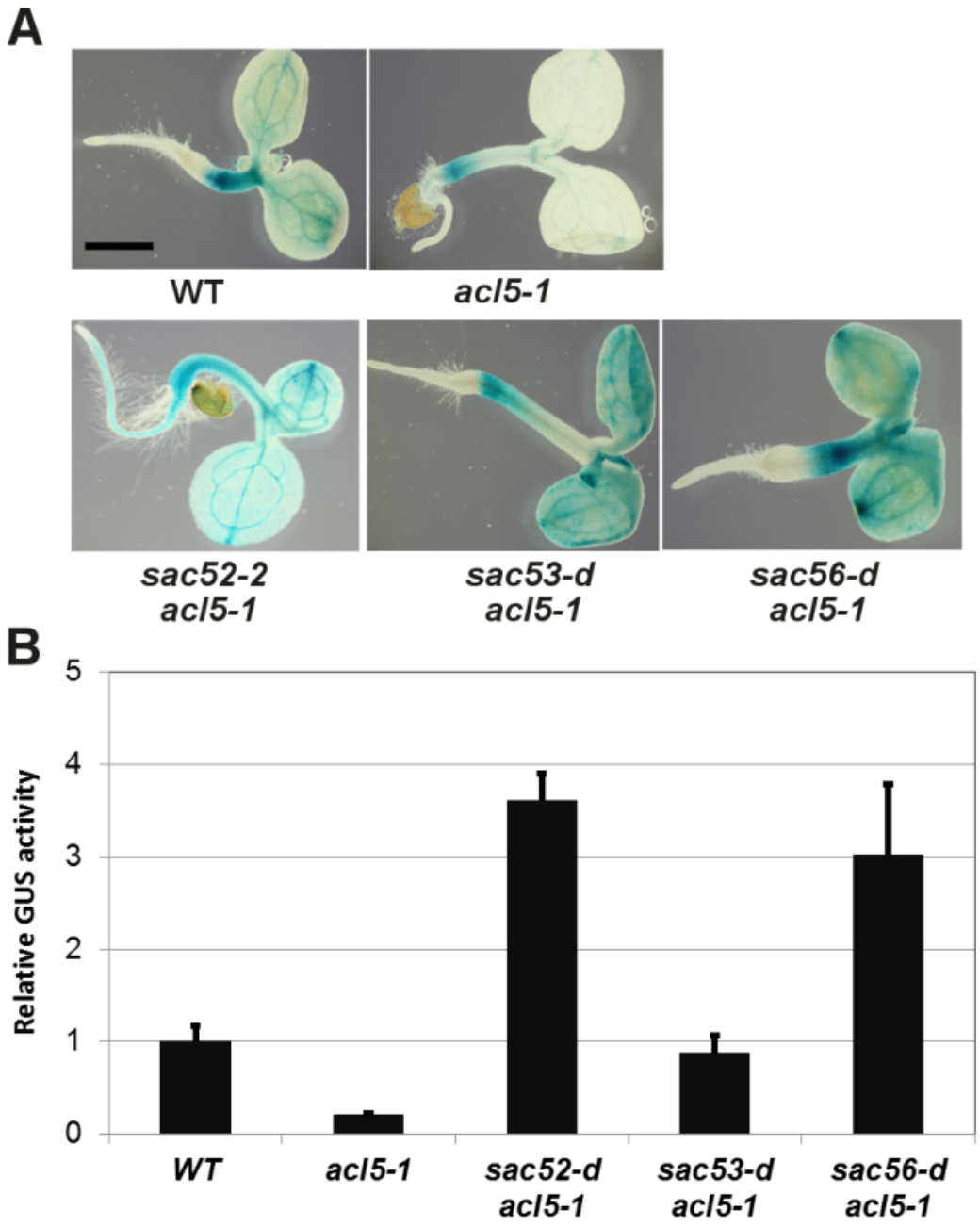

Fig. 16

Effects of *sac53-d* and *sac56-d* on the SAC51-GUS fusion gene expression.

sac acl5-1 seedlings carrying the *GUS* reporter gene fused with the CaMV 35S promoter and the *SAC51* 5′ leader region were grown for 3 days in MS solutions, and stained (A) or assayed (B) for GUS activity. The scale bar indicates 1 cm. Error bars indicate S.D. values of three independent experiments.

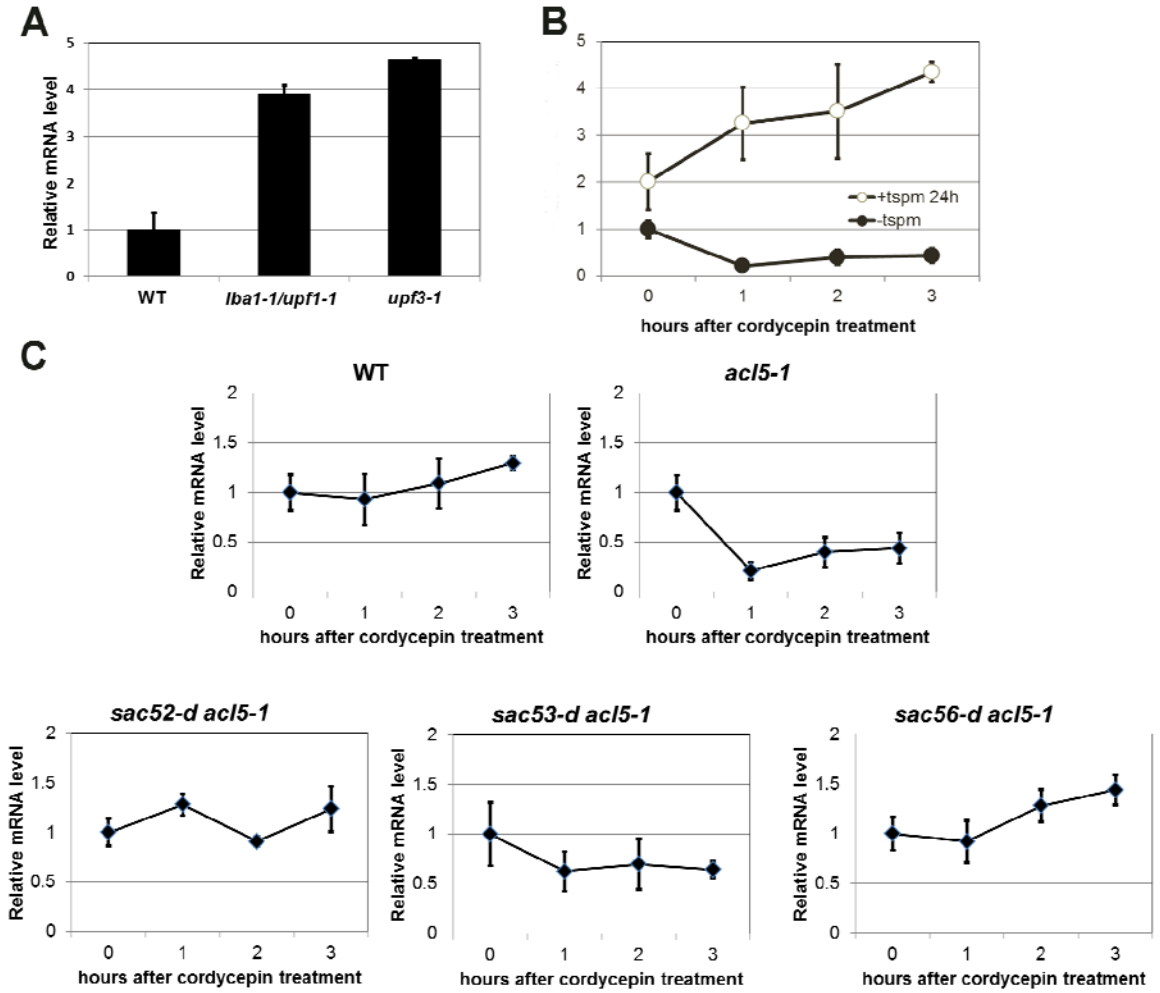

Fig. 17

Effects of thermospermine and *sac* mutations on the *SAC51* mRNA stability.

(A) Relative mRNA level of *SAC51* mRNA in *upf1-1* and *upf3-1*. (B) Time-course assays of *SAC51* mRNA stability in *acl5-1*. Seedlings were grown for 10 days, and treated with 0.6 mM cordycepin for indicated periods after mock or 0.1 mM thermospermine treatment for 1 hour. Closed circles and open circles indicate mock and thermospermine treatment, respectively. (C) Time-course assays of mRNA stability in each *sac acl5* double mutant. Seedlings were grown for 10 days, and treated with 0.6 mM cordycepin for indicated periods. mRNA levels were set to 1 at 0 hour and normalized to the *UBQ10* mRNA. Error bars indicate S.D. values of three independent experiments.

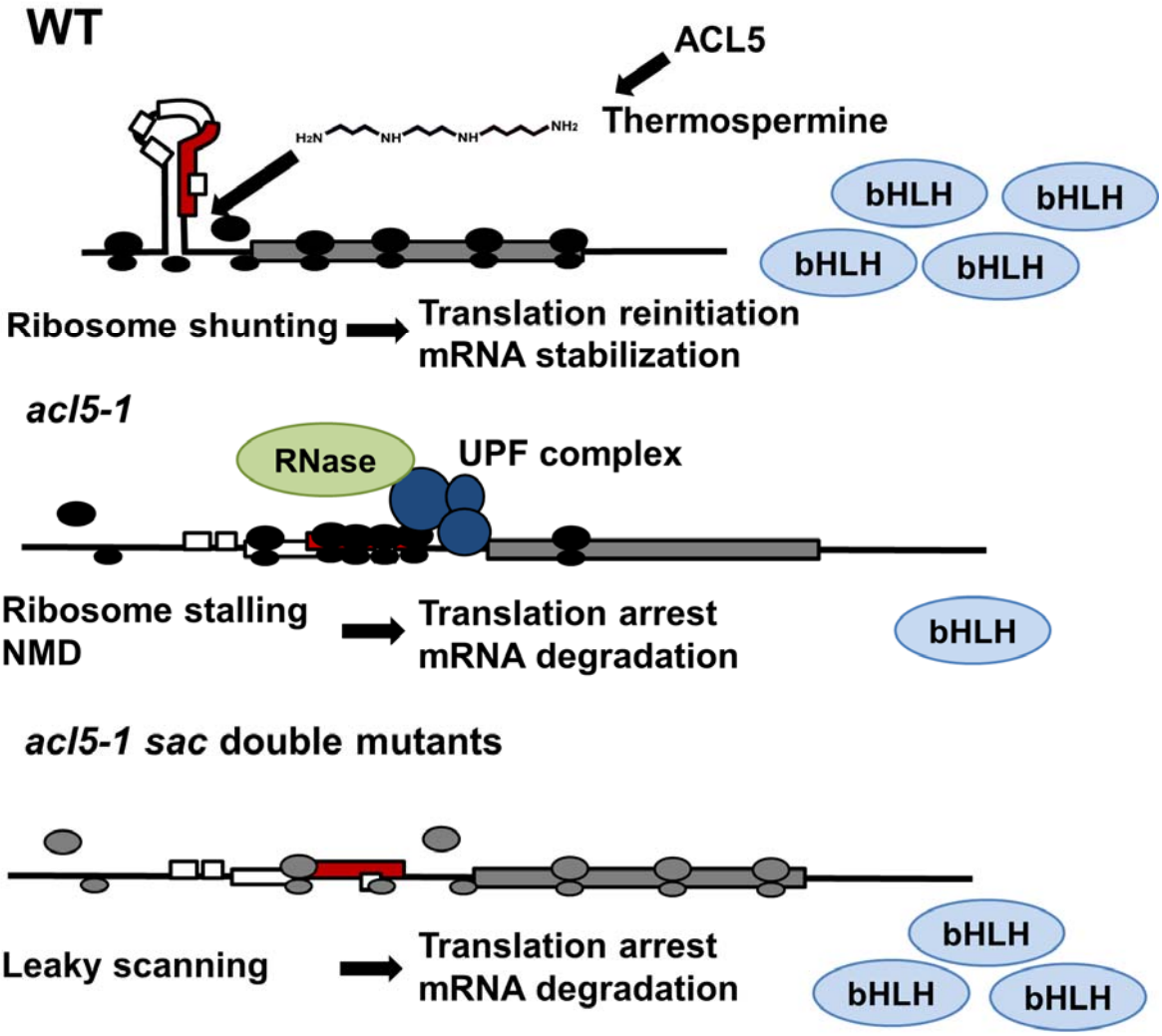

Fig. 18

A working model for actions of thermospermine and *sac* mutations in *SAC51* translation

Red box indicates *SAC51* 4 th uORF, white boxes indicate other uORFs of *SAC51* mRNA, and grey box indicates main ORF encoding a bHLH transcription factor. Black circles represent small and large subunits of the wild-type ribosome. Grey circles indicate large and small subunits containing mutated ribosomal protein of *sac* mutants (*sac52-d*, *sac53-d*, or *sac56-d*) ribosome. The fourth upstream ORF of *SAC51* has a negative effect on translation of the *SAC51*, probably by stalling the ribosome and NMD (Imai et al., 2006 and this study). I propose that thermospermine produced by the action of ACL5 causes structure changes in 5'-UTR of *SAC51* mRNA, promotes the ribosome shunting, and enhances translation reinitiation of the *SAC51* main ORF and the inhibition of NMD. Furthermore, mutations of ribosomal proteins (*sac52-d*, *sac53-d*, and *sac56-d*) affect the ribosome to destabilize the ribosome stalled on the upstream ORF, and allow the leaky scanning to reach the start codon of the main ORF more efficiently.

Table 2. Primers for Mapping of *sac56-d*.

Marker name		Sequence	Restriction enzyme
T22N4	F	5'-GACTGTTTGACTCCAAGTG-3'	-
	R	5'-TCGTTACGAACCTCTGGTA-3'	
F3L24	F	5’-GAGCAATGATGGTTAGCAG-3’	-
	R	5’-CGTAGCCCCTCACAAAAAA-3	
2.88	F	5’-CAGGGTTGTTGTTCAGG-3	*Hae*III
	R	5’-CTCAGATTGACGAGTTGC-3	
2932/F11F8-1	F	5'-TGTTTGAGACTCGTCACAAG-3'	*Msp*I
	R	5'-ATACCTGGTTCCAGTTCCTT-3'	
3.06/F8A24-1	F	5'-GTCACGGATAATCTTCTTGG-3'	*Bsp*HI
	R	5'-GACCAATATGGTATGGGTTC-3'	
3.08	F	5'-GGAAGCATTGACTTAGAGTG-3'	*Bsp*HI
	R	5'-GACATGCATTGCAATTGGGT-3'	
MNSOD	F	5'-CTCCCAAACATCTATACCCACCAG-3'	*Hpy*CHIV
	R	5'-TTACGCTTCCTGATCTTCCTTACG-3'	
MZN24	F	5'-CCGAACCGAAATCAACTGTACC-3'	-
	R	5'-CTGAACGAGAGGAACATGGAGT-3'	

Table 3. Primers for Genotyping.

Gene name		Sequence	Restriction enzyme
acl5-dCAPS	F	5'-GGAGGTGAAGGCTCTGCTGCTCGA -3'	*Xho*I
	R	5'-TCGGATCCCAGAAAGCATCGCTGTTAAC-3'	
sac52-dCAPS	F	5'-TGCGAGGTGTTACCGTCAGATCTAG-3'	*Xba*I
	R	5'-CCATGACACCAAATGGACACAG-3	
sac53-dCAPS	F	5'-GAGCACTGTGGCTGTATCACC-3'	*Bgl*II
	R	5'-CTCAACAATGCTCTTGCTCTCAAGATC -3'	
sac56-dCAPS	F	5'-TTGCTCAGATTATGGTCCGA-3'	*Ava*II
	R	5'-GACATTTGAATTCGGTTTGAGCTTC-3'	
RACK1A-5'	-	5'-GGCATCTCCAGACACCGAAA-3'	-
RACK1A-3'	-	5'-GCAGAGAGCAACGACAGC-3'	-
pBI-LB	-	5'-AACCAGCGTGGACCGCTTGCTG-3'	-

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