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The dwarf phenotype of the *Arabidopsis acl5* mutant is suppressed by a mutation in an upstream ORF of a bHLH gene

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Loss-of-function mutants of the *Arabidopsis thaliana ACAULIS 5* (*ACL5*) gene, which encodes spermine synthase, exhibit a severe dwarf phenotype. To elucidate the *ACL5*-mediated regulatory pathways of stem internode elongation, we isolated four *suppressor of acaulis* (*sac*) mutants that reverse the *acl5* dwarf phenotype. Because these mutants do not rescue the dwarfism of known phytohormone-related mutants, the *SAC* genes appear to act specifically on the *ACL5* pathways. We identify the gene responsible for the dominant *sac51-d* mutant, which almost completely suppresses the *acl5* phenotype. *sac51-d* disrupts a short upstream open reading frame (uORF) of *SAC51*, which encodes a bHLH-type transcription factor. Our results indicate that premature termination of the uORF in *sac51-d* results in an increase in its own transcript level, probably as a result of an increased translation of the main ORF. We suggest a model in which *ACL5* plays a role in the translational activation of *SAC51*, which may lead to the expression of a subset of genes required for stem elongation.

KEY WORDS: *Arabidopsis thaliana*, Polyamine, Spermine, Stem elongation, Upstream ORF

INTRODUCTION

The polyamines, including putrescine, spermidine and spermine, are ubiquitous components of living organisms and are essential primary metabolites for normal growth and development (Tabor and Tabor, 1984; Heby and Persson, 1990). These low-molecular-weight compounds are positively charged at physiological pH and bind to negatively charged molecules, e.g. nucleic acids, acidic phospholipids and various types of proteins (Cohen, 1998). The biosynthesis of putrescine occurs from the processing of ornithine and/or arginine, and is regulated by the enzymes ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), respectively. Subsequent reactions convert putrescine into spermidine and spermine, and these are catalyzed by spermidine and spermine synthases, which add propylamino groups from decarboxylated *S*-adenosylmethionine, catalyzed by *S*-adenosylmethionine decarboxylase (SAMDC) (Pegg, 1988).

Studies of higher plants have shown that polyamines play important roles in a wide range of developmental processes, such as embryogenesis, floral development, fruit ripening, senescence, and response to environmental stresses (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1990; Bouchereau et al., 1999). The *acaulis 5* (*acl5*) mutant of *Arabidopsis thaliana* is defective in a spermine synthase and exhibits a severe dwarf phenotype, suggesting that spermine is a novel regulator of stem elongation (Hanzawa et al., 2000). Spermine has also been identified as a signal mediator of the defense responses in tobacco (Takahashi et al., 2003; Takahashi et al., 2004). Moreover, transgenic potatoes that express the antisense *SAMDC* gene under the control of the CaMV 35S promoter with a duplicated enhancer region exhibit stunted growth with highly branched stems and short internodes (Kumar et al., 1996). Overexpression of the oat *ADC* gene in transgenic tobacco plants results in short internodes, thin stems and leaves, leaf necrosis and short roots (Masgrau et al., 1997). However, the molecular mechanisms by which polyamines control plant growth remain unknown.

The *Arabidopsis* genome has two genes encoding spermidine synthase, *SPDS1* and *SPDS2*, and two genes encoding spermine synthase, *ACL5* and *SPMS* (Hanzawa et al., 2002; Panicot et al., 2002) (see Fig. S1 in the supplementary material). The *spds1 spds2* double loss-of-function mutant shows an embryo lethal phenotype (Imai et al., 2004b). The *spms* mutant shows no aberrant phenotype, whereas *acl5 spms* double mutants contain no endogenous spermine but display a dwarf phenotype that is identical to the *acl5* single mutant (Imai et al., 2004a). The *ACL5* gene is upregulated by auxin, whereas the *SPMS* gene is responsive to abscisic acid (Hanzawa et al., 2002). Moreover, SPMS interacts with SPDS1 and SPDS2 to form 'metabolon' complexes, whereas ACL5 does not interact with either of these proteins (Panicot et al., 2002). Measurement of the polyamine levels in *acl5* and *spms* mutants has also revealed that *SPMS* is a principal contributor to spermine biosynthesis in vivo (Imai et al., 2004a). These findings thus suggest that *ACL5* is specifically required for stem elongation.

To further elucidate the role of *ACL5* during stem elongation, we identified extragenic suppressors of the *acl5* mutant and designated these as *suppressor of acaulis* (*sac*) mutants. We show that the *sac51-d* mutation disrupts a short upstream open reading frame (uORF) of the *SAC51* gene, which encodes a basic helix-loop-helix (bHLH) transcription factor. Our results suggest that *ACL5* is involved in the translational control of the *SAC51* gene.

MATERIALS AND METHODS

Plant materials and growth conditions

The Landsberg *erecta* (L*er*) ecotype of *Arabidopsis thaliana* was used in all experiments except for those involving *axr2-1* (Timpte et al., 1994), *dim* (Takahashi et al., 1995), *spms-1* (Imai et al., 2004a) and transgenic lines from the Columbia (Col-0) ecotype. The *axr2-1* and *gai-1*

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(Koornneef et al., 1985) mutants were obtained from the Arabidopsis Biological Resource Center. *acl5-1* of the Col-0 ecotype was generated by more than seven crosses of the original *acl5-1* strain of the L*er* ecotype (Hanzawa et al., 1997) to wild-type Col-0.

Plants were grown under continuous fluorescent light at 22°C on rockwool bricks supplemented with vermiculite, or on 0.8% (w/v) agar plates containing MS salts (pH 5.8) and 3% sucrose, after surface sterilization of seeds. Heat-shock treatments of plants were performed as described previously (Matsuhara et al., 2000).

Mutagenesis and screens for *sac* mutants

Mutagenesis of the *acl5-1* seeds with ethylmethane sulfonate (EMS) was performed as described previously (Takahashi et al., 1992). Briefly, approximately 20,000 *acl5-1* seeds were surface-sterilized, hydrated, treated with 0.2% EMS (Sigma, St Louis, MO, USA) for 14 hours and washed extensively. The M1 plants were then divided into 10 pools and self-pollinated. Approximately 2000 M2 seeds from each pool were grown for suppressor screening. Putative suppressors showing recovery from the *acl5-1* dwarf phenotype were used for further analysis after being backcrossed three times to *acl5-1* of either the L*er* or Col-0 ecotypes.

Mapping and cloning

Each of the *sac acl5* mutants of the L*er* ecotype was crossed to *acl5-1* of the Col-0 ecotype. Genomic DNA was extracted from F2 plants showing the *acl5* phenotype and analyzed for co-segregation with respect to cleaved amplified polymorphic sequence (CAPS), simple sequence length polymorphism (SSLP) and single nucleotide polymorphism (SNP) markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). These markers were derived from the Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org). The CAPS markers MDC12Dra, MHJ24Dde, MSJ1Eco and MSJ1Hha were developed for mapping the *SAC51* locus by using the primer sequences shown in Table 1. DNA sequences were determined from PCR products using a 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Genotyping

sac dim double mutants were identified by initially crossing the *sac acl5* mutant with the *dim* strain. In the F2 generation, plants that are homozygous for the *dim* allele (*dim/dim*) and heterozygous for the *acl5-1* allele (*acl5-1*/+) were then selected based on both the *dim* phenotype and the dCAPS (Neff et al., 1998) of the *ACL5* locus. The dCAPS primers used for genotyping the *ACL5* locus were ACLdF and ACLdR, which produce a single PCR fragment from *acl5-1* and two cleaved PCR fragments from *ACL5*, after *Xho*I digestion and resolution on an 8% acrylamide gel. All of the selected F2 plants (*dim/dim acl5-1*/+) were then subjected to growth assays and were further crossed to *acl5-1* to determine the *SAC* genotype. F2 plants that segregated with no progeny of the *acl5* phenotype in the F3 generation of this testcross were consequently identified as homozygous *sac* mutants and their growth data were calculated. The genotypes of the *SPMS* locus in the *sac acl5 spms* mutants were confirmed by PCR with *SPMS*-specific primers (Imai et al., 2004a). For primer sequences, see Table 2.

Gene expression analyses

Total RNA was extracted from 12-day-old seedlings grown on MS agar plates or from flower buds with apical inflorescence meristems of 6-week-old plants. RNA gel blot analyses with *EXGT-A1*, *γ-TIP*, *ACL*, and *SPMS*, were performed as described previously (Hanzawa et al., 1997; Hanzawa et al., 2002). A gene-specific probe for *SAC51* was prepared by PCR from the wild-type genomic DNA using 51F1 and 51R1 primers.

Reverse transcription reactions were done with 1 μg of DNase-treated total RNA and 2.5 pmol oligo(dT) primer in 20-μl reactions using an RNA LA PCR Kit (Takara, Kyoto, Japan), according to the manufacturer's protocol. Quantitative PCR was performed in a DNA Engine Opticon2 System (Bio-Rad, Hercules, CA) using gene-specific primers. Triplicate PCR reactions were averaged. The primers 51F2 and GUSR were used for detecting expression of *SAC51-GUS* and *sac51-d-GUS* chimeric transcripts. Relative transcript levels in all samples were normalized using *ACTIN8* (An et al., 1996). For primer sequences, see Table 2.

Plasmid construction and plant transformation

For recapitulation of the *sac51-d* phenotype, a 4.1-kb genomic fragment encompassing from 990 bp upstream of the *SAC51* transcription start site to 723 bp downstream of the *SAC51* stop codon was amplified from the *sac51-d* allele with 51F3 and 51R2 primers, digested with *Bgl*II, and cloned into the *Bam*HI site of pBI101 (Clontech, Palo Alto, CA), resulting in pSAC51R. For generating the *SAC51* 5′-leader deletion construct, the 990-bp *SAC51* promoter fragment was amplified with primers 51F4 and 51R3, digested with *Cla*I and *Xba*I, and inserted into the *Cla*I/*Xba*I-digested pBI101, resulting in pSAC51pro. The *SAC51* coding sequence was amplified from genomic DNA using 51F5 and 51R1 primers, subcloned into pGEM-T Easy (Promega, Madison, WI), and further transferred as a *Spe*I-digested fragment to the *Xba*I-digested pSAC51pro, resulting in pSAC51Δ5′. For heat shock-inducible *SAC51* expression, the same *Spe*I-digested *SAC51* fragment was transferred to the heat-shock cassette Ti-vector pTT101 (Matsuhara et al., 2000), resulting in pHS-SAC51Δ5′. For heat shock-inducible *SPMS* expression, the *SPMS* cDNA was amplified with SPMSF and SPMSR primers, subcloned into pGEM-T Easy, and further transferred as a *Sac*I fragment to pTT101, resulting in pHS-SPMS. These Ti constructs were used to transform *acl5-1* in the Col-0 background. The *acl5-1* mutant carrying the *HS-ACL5* construct was previously described (Hanzawa et al., 2000).

For GUS expression analysis, the 990-bp *SAC51* promoter fragment and the 5′-leader region of either the wild-type *SAC51* or *sac51-d* transcripts were amplified by PCR with 51F3 and 51R4 primers, digested with *Bgl*II, and cloned into the *Bam*HI site of pBI101. The construct that contains a point mutation in the *SAC51* uORF (*sac51-C549A-GUS*) was generated by a two-step mutagenesis protocol. PCR amplification was first performed using 51F3/mut-C549AR and mut-C549AF/51R4 primer pairs. The PCR products were subjected to a second round of amplification with 51F3 and 51R4, and cloned into pGEM-T Easy. After checking the sequence, the *Bgl*II-digested fragment was cloned into pBI101 as described above.

Transformation of *Arabidopsis* was carried out using the floral dip method (Bechtold and Pelletier, 1998) with the *Agrobacterium* strain C58C1. Transformants were selected in MS agar plates containing 50 μg ml^{-1} kanamycin. Independent transgenic lines that segregated 3:1 for the kanamycin-resistance marker in the T2 generation were further selected to isolate progeny that were homozygous for the transgene.

Microscopy

Inflorescence stems of 6-week-old plants were fixed overnight in 50% ethanol, 5% formaldehyde and 5% acetic acid. The samples were then dehydrated through an ethanol series and embedded in Technovit 7100

Table 1. CAPS markers designed for this study

Marker	Primer sequences	Digestion*
MDC12Dra	F, 5′-AACTAAAATGAAACAGAACT-3′; R, 5′-TAGAGTTAATGAAAGGAAAA-3′	*Dra*I
MHJ24Dde	F, 5′-TGGGACTACAATGCTATTTC-3′; R, 5′-AGGGTTCGAAGTTATTGAAG-3′	*Dde*I
MSJ1Eco	F, 5′-GAGTGAAGAGCGAAACTCTC-3′; R, 5′-TTACACTTACACCAAAGAAA-3′	*Eco*RI
MSJ1Hha	F, 5′-AAAGAGGAACATGATGAGAG-3′; R, 5′-ACTGCTTTGTACTCTTTGAG-3′	*Hha*I

*Restriction enzymes used for digestion of the PCR products.
F, forward; R, reverse.

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Table 2. Primer sequences used in this study

Primer names	Primer sequences
ACLdF	5′-GGAGGTGAAGGCTCTGCTGCTCGA-3′
ACLdR	5′-GTTACAGAAAGCATCGCTGTTAAC-3′
SPMSF	5′-GAGAGCTCGGAATAGGTTTGGTA-3′
SPMSR	5′-TCCTCTTCAAGAGTTCTACAAAG-3′
51F1	5′-AAGAGCATGCCTCTGGATAAGAG-3′
51F2	5′-GGTATCTGTCTACTTTCTTC-3′
51F3	5′-AGATCTTAAAATTCCCGTGCAATTC-3′
51F4	5′-TATCGATTCCCGTGCAATTCTTTA-3′
51F5	5′-ACTAGTTCCCTTGAGCTAAACTG-3′
51R1	5′-ATACAGCTAAAAAGCTGGTG-3′
51R2	5′-AGATCTGGTGTTGATCATCCAC-3′
51R3	5′-TTCTAGAAAGTGACCAACGAACA-3′
51R4	5′-AGATCTAGAGGCATGCTCTTAG-3′
HB8F	5′-AGCGTTTCAGCTAGCTTTTGAG-3′
HB8R	5′-CAGTTGAGGAACATGAAGCAGA-3′
CNAF	5′-GAAACTCCACATGTGCAAGA-3′
CNAR	5′-TCTCTCATACGAAACTGGTC-3′
IFLF	5′-GTGAGAGAAGCAGTGACAGT-3′
IFLR	5′-CAGCAGGACTATTCGCATCT-3′
PHBF	5′-ATTGCCCTCTGGCGTTTTCT-3′
PHBR	5′-TGCTATAGAAAGGAGTCCT-3′
PHVF	5′-GCTCCTTTTACCTCTTTCCATC-3′
PHVR	5′-CGCAATCGAGAGAAGATTA-3′
ACT8F	5′-TGAGCCAGATCTTCATCGTC-3′
ACT8R	5′-TCTCTTGCTCGTAGTCGACA-3′
GUSR	5′-TCACGGGTTGGGGTTTCTAC-3′
mut-C549AF	5′-TTATGGTGTGCAAATCACCTGGTAAGACA-3′
mut-C549AR	5′-AGGTGATTTGCACACCATAACTTTGAGGA-3′

resin (Heraeus Kulzer, Wehrheim, Germany). Sections (8 μm) were stained with 0.1% Toluidine blue for 15 seconds.

GUS assays

Histochemical and fluorometric GUS assays were performed as described previously (Jefferson et al., 1987). For histochemical analysis, samples were prefixed in 90% acetone at room temperature for 20 minutes. Protein content was determined using the Bradford assay (Bio-Rad) to compare activity to protein units, and each experiment was repeated three times.

RESULTS

Four *sac* mutants suppress the *acl5* phenotype to different degrees

Mutations that suppress the dwarf phenotype of the *acl5* mutant were identified by screening for tall individuals among M2 plants descended from EMS-mutagenized homozygous *acl5-1* seeds. A total of four putative suppressors were isolated from approximately 20,000 M2 plants and designated as *suppressor of acaulis* (*sac*) *51-54*. After establishing homozygous *sac acl5-1* plants by self-pollination, we crossed each of them to the wild-type L*er* strain and confirmed the segregation of plants showing the *acl5* phenotype in each F2 population. Thus, all four *sac* mutants appeared to represent extragenic suppressors of *acl5-1* and not a reversion of the *acl5-1* allele. We further backcrossed these *sac acl5-1* mutants to the *acl5-1* single mutant. F1 plants from the cross between *acl5-1* and *sac51 acl5-1* were indistinguishable from *sac51 acl5-1*, whereas those from the cross of *acl5-1* to *sac52*, *sac53* or *sac54* in the *acl5-1* background displayed an intermediate stature when compared with the parental lines (Fig. 1). These results indicate that *sac51* is completely dominant, whereas *sac52*, *sac53* and *sac54* are semi-dominant. Mapping experiments of each *sac* locus revealed that *SAC51* is located on chromosome V and *SAC54* is on chromosome III. *SAC52* and *SAC53* were mapped to approximately 6.6 cM and 13.3 cM from the SSLP marker nga63 on chromosome I,

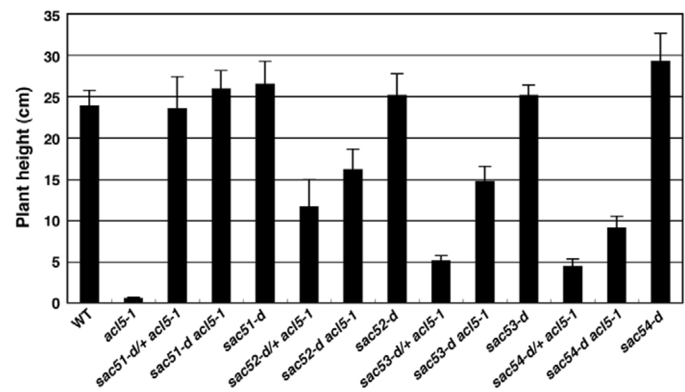

Fig. 1. Height comparisons between wild-type (WT), *acl5*, *acl5* heterozygous for *sac* (*sac*/+), *acl5* homozygous for *sac*, and *sac* plants. The heights were measured in 6-week-old plants. Bars show mean±s.d. (n=10).

respectively (see Fig. S1 in the supplementary material). Hereafter, we refer to these dominant or semi-dominant *sac* alleles as *sac51-d* to *sac54-d*.

As shown in Fig. 2A, *sac51-d acl5-1* plants are wild type in appearance, whereas *sac52-d acl5-1*, *sac53-d acl5-1* and *sac54-d acl5-1* plants have sizes of approximately 68%, 62% and 38% of the wild-type height, respectively. Mature *acl5-1* plants do not only display a reduction in their height but also have reduced lengths in their leaf blades, petioles and pedicels, and in their stem diameters (Hanzawa et al., 1997). We found that the restoration of these organ sizes is paralleled by that of the plant height in *sac51-d acl5-1* and *sac52-d acl5-1* double mutants (Fig. 2A, Table 3). The *sac53-d* allele is more effective in restoring the stem length (~61% recovery), than in restoring the stem diameter (~31% recovery), rendering *sac53-d acl5-1* plants more slender than wild-type plants. Interestingly, in *sac54-d acl5-1* plants, the pedicel length is fully restored but the stem length is only partially restored (~39% recovery). Microscopic observations of stem longitudinal sections revealed that the recovery of plant height is attributable to cell lengths in all four classes of *sac acl5* mutants (Fig. 2B, Table 3). Our genetic segregation data revealed that all *sac* single mutants show no obvious phenotype in the presence of the wild-type *ACL5* gene (data not shown).

Vascular phenotype of *sac acl5* mutants

acl5-1 shows an overproliferation of lignified xylem tissues in the stem (Hanzawa et al., 1997). This phenotype is suppressed in *sac acl5* mutants, in accordance with the recovery of the plant height (Fig. 2C). The abnormalities in xylem development and lignin accumulation in *acl5-1* are reminiscent of those observed in transgenic *Arabidopsis* plants that constitutively express *ATHB8*, a gene encoding a class III homeodomain-leucine zipper (HD-Zip III) transcription factor (Baima et al., 2001). The HD-Zip III genes have been implicated in the regulation of vascular differentiation (Baima et al., 1995; Baima et al., 2001; Zhong et al., 1999; Ohashi-Ito and Fukuda, 2003), leaf polarity (McConnell et al., 2001) and meristem initiation (Otsuga et al., 2001; Green et al., 2005). We prepared RNA samples from 12-day-old seedlings of wild type, *acl5*, and four classes of *sac acl5* plants, and examined the transcript levels of all five HD-Zip III genes (Prigge et al., 2005) by quantitative real-time RT-PCR. Our results revealed that the expression of each of these

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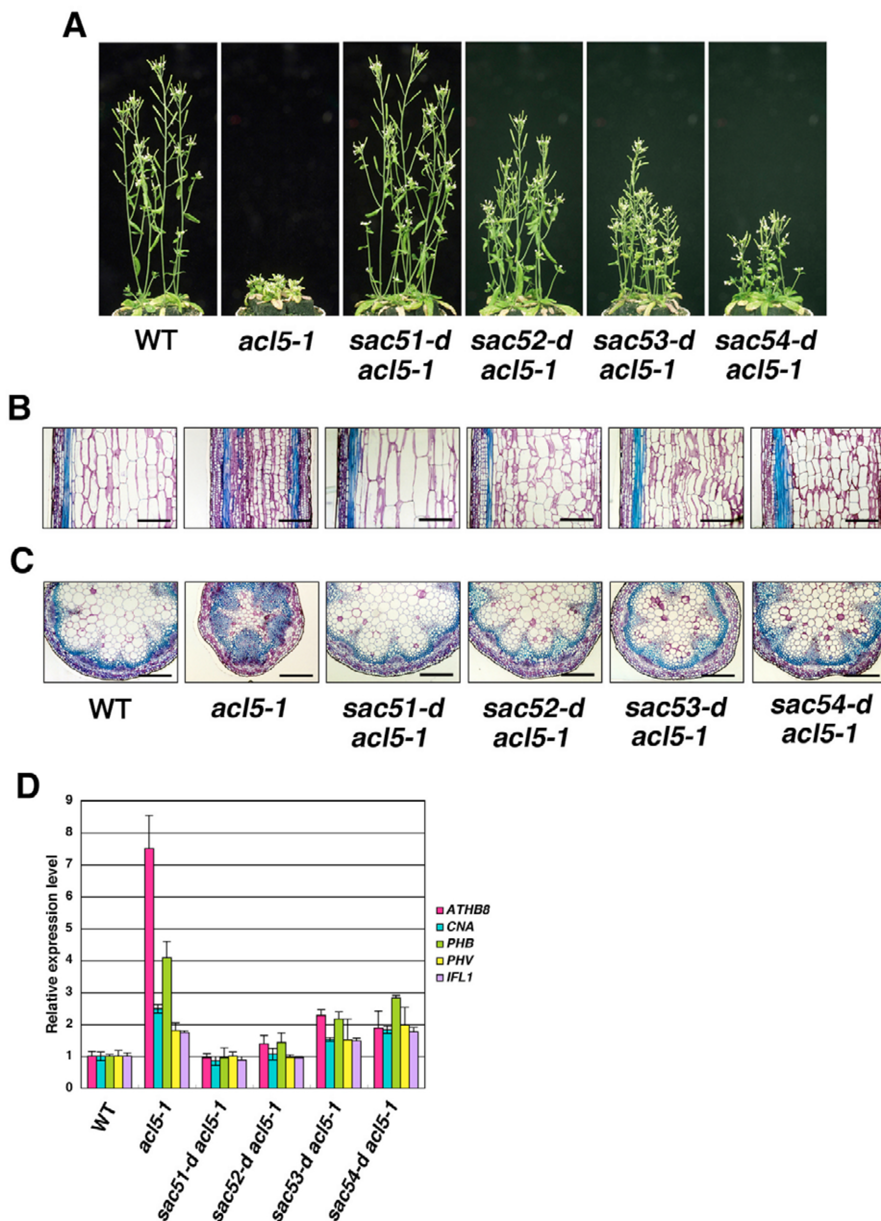

Fig. 2. Morphological phenotypes of *sac acl5* mutants. (**A**) From left to right, 6-week-old wild-type (WT), *acl5* and *sac acl5* plants. (**B**,**C**) Longitudinal (B) and transverse (C) sections of the first internode of wild-type (WT), *acl5* and *sac acl5* inflorescence stems. Scale bars: 200 μm. (**D**) Quantitative RT-PCR analysis of the HD-Zip III class genes. Total RNA was prepared from whole seedlings of 12-day-old wild-type (WT), *acl5* and *sac acl5* plants. Levels of the *ACTIN8* (*ACT8*) transcript were used as a reference; values are expressed as ratios to the transcript level of each gene in the wild-type seedlings. Bars show mean±s.d. (n=3).

genes is upregulated in *acl5-1* but is restored to normal levels in *sac acl5* mutants, in parallel with the degree of recovery of plant height during the adult stage of growth (Fig. 2D).

sac mutations affect the genes with altered expression in *acl5*

A previous study revealed that *acl5-1* shows reduced expression levels of *EXGT-A1*, which encodes the cell wall enzyme endoxyloglucan transferase, and of *γ-TIP*, which encodes a vacuolar aquaporin, tonoplast intrinsic protein (Hanzawa et al., 1997). To examine the effect of each *sac* allele on the expression of these genes, northern blot analysis was performed with total RNA isolated from flower buds with apical inflorescence meristems of 6-week-old plants. The levels of both the *EXGT-A1* and *γ-TIP* transcripts were restored in *sac acl5* mutants (Fig. 3). We also examined *acl5-1* transcript levels in each *sac acl5* mutant. In contrast to *EXGT-A1* and *γ-TIP*, the *acl5-1* transcript level is upregulated in *acl5-1*, probably as a result of a negative-feedback control of *ACL5* expression (Hanzawa et al., 2000). The *acl5-1* transcript levels were restored in accordance with the degree of recovery of the plant height in *sac acl5* plants (Fig. 3). The recovery in *acl5-1* transcript levels was also detected at the seedling stage, prior to the manifestation of morphological phenotypes (data not shown). *SPMS* transcript levels were not affected in any of the *sac acl5* mutants.

sac mutants do not suppress hormone-related dwarf phenotypes

To determine whether *sac* mutations are general suppressors of hormone-related dwarf phenotypes or not, the *sac* mutants were crossed to an auxin-resistant mutant, *axr2*, a GA-insensitive mutant, *gai*, and a BR-requiring mutant, *dim*. Because *axr2-1* and *gai-1* alleles are dominant, we crossed their respective mutants with *sac acl5* and measured the plant height in the F1 generation. *sac dim* double mutants were identified as homozygotes for both alleles. Our results revealed that none of the *sac* alleles suppresses the dwarf phenotypes of *axr2*, *gai* and *dim* mutants (Fig. 4A).

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Table 3. Suppression of the *acl5* phenotype by *sac* mutations

	Wild type	*acl5-1*	*sac51-d acl5-1*	*sac52-d acl5-1*	*sac53-d acl5-1*	*sac54-d acl5-1*
Aerial organ growth*						
Length of leaf blade (mm)†	19.2±2.4	8.8±1.7	20.3±4.3	18.3±1.0	14.0±2.5	15.5±2.9
Length of petiole (mm)†	5.4±0.9	2.8±1.0	6.0±0.8	4.5±0.6	4.3±0.5	3.5±0.6
Length of pedicel (mm)‡	6.0±0.7	1.0±0.2	6.5±0.6	4.5±0.6	3.0±0.8	7.5±1.7
Diameter of stem (mm)§	0.88±0.07	0.46±0.05	0.87±0.01	0.81±0.06	0.59±0.09	0.63±0.05
Stem cell length¶						
Length of epidermal cells (μm)¶	177.1±47.4	21.4±4.5	136.1±56.5	53.0±12.8	65.1±40.6	38.6±24.2
Width of epidermal cells (μm)¶	13.4±2.0	10.5±0.9	13.4±1.6	10.7±0.9	11.7±1.9	14.7±3.1
Length of pith cells (μm)¶	249.7±43.5	71.3±22.3	269.8±54.1	107.9±25.6	134.4±22.9	99.3±28.4
Width of pith cells (μm)¶	59.1±8.3	30.1±4.5	53.1±14.7	62.6±8.3	45.4±6.6	48.4±9.6

*Measurements of 6-week-old plants. Each value represents the average of at least 10 plants (±s.d.).
†The fifth rosette leaves were measured.
‡Samples were taken 10 days after pollination.
§Samples were collected from the middle of the first internode.
¶Epidermal cells and pith cells were sampled from the longitudinal sections of the first internodes of 6-week-old plants and the length or width of at least 20 cells of five random areas of each section were measured (±s.d.).

acl5-1 spms-1 plants have no detectable levels of endogenous spermine but are morphologically indistinguishable from *acl5-1* (Imai et al., 2004a). To examine the effect of *sac* alleles on stem elongation in a background of complete spermine depletion, we generated *sac acl5 spms* triple mutants. All four of the *sac* mutants suppressed the *acl5* phenotype to a similar degree in either the presence or absence of *SPMS* (Fig. 4B).

SAC51 encodes a bHLH protein

We chose the *sac51-d* allele for further analysis. Fine mapping experiments placed the *SAC51* locus within a 60-kb region of the P1 clone MSJ1 (Fig. 5A). All of the genes in this region were amplified by PCR from homozygous *sac51-d* plants and their sequences were compared with those of the wild-type L*er*. We detected a single C-to-T mutation in the *At5g64340* gene (Fig. 5B). A genomic fragment encompassing from 990 bp upstream of the transcription start site to 723 bp downstream of the stop codon of *At5g64340* was cloned from *sac51-d* plants and introduced into *acl5-1* in the Col-0 background by *Agrobacterium*-mediated transformation. The resulting transgenic plants displayed the wild-type phenotype in four independent lines (see Fig. S2 in the supplementary material), confirming that *At5g64340* is indeed the *SAC51* gene.

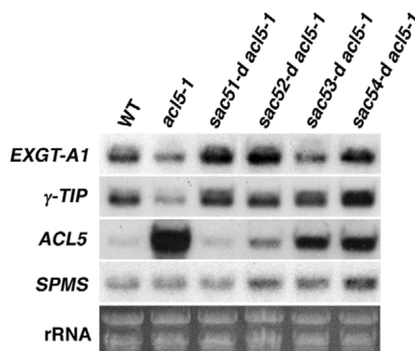

Fig. 3. Northern analysis of *EXGT-A1*, *γ-TIP*, *ACL5* and *SPMS* expression levels. Total RNA was prepared from apical meristems and flower buds of 6-week-old wild-type (WT), *acl5,* and *sac acl5* plants. Each lane contains 10 μg of total RNA. rRNA is shown as a loading control.

The full-length *SAC51* cDNA in the GenBank database is 2,472 bp in length and is separated by three introns. The longest ORF encodes a protein of 348-amino acids with an estimated molecular mass of 37.8 kDa. The SAC51 protein contains a basic helix-loop-helix (bHLH) domain in its C-terminal half (Fig. 5C) and has been designated as AtbHLH142 in the compilation of Toledo-Ortiz et al. (Toledo-Ortiz et al., 2003). SAC51 shows a high sequence similarity over the entire length of the protein (57.3% identity) to AtbHLH143 (At5g09460). The bHLH sequence homologies between SAC51, At5g09460 and three known bHLH proteins in *Arabidopsis* are shown in Fig. 5D. Most known plant bHLH proteins also exhibit homology outside of the bHLH domain, such as in the MYB-interacting domain of the R proteins (Goff et al., 1992; Abe et al., 1997) or the PAS domain of PIF3 (Kay, 1997). However, such domains are not conserved in SAC51.

The *SAC51* cDNA sequence reveals that the *SAC51* transcript includes an approximately 870-nucleotide 5′-leader region with five overlapping uORFs (Fig. 5B,C). The *sac51-d* allele has a C-to-T nucleotide exchange that creates a premature stop codon in the fourth uORF. If the fourth uORF is recognized by a scanning ribosome and translated, it is predicted to encode a 53-amino-acid peptide in wild-type plants but only a 3-amino-acid peptide in *sac51-d* mutants. The *At5g09460* transcript also has a long 5′-leader sequence with five overlapping uORFs. The polypeptide sequence deduced from the fourth uORF of *SAC51* shares 69.8% amino acid identity with that of *At5g09460*.

Northern analysis revealed that *SAC51* shows high expression in stems, roots and flowers, but little or no expression in siliques (Fig. 6A). The *sac51-d* transcript level was markedly higher in *sac51-d acl5-1* and *sac51-d* seedlings than was the *SAC51* transcript level in either wild-type or *acl5-1* seedlings (Fig. 6B; data not shown). Other *sac* mutations did not affect the *SAC51* transcript level (Fig. 6B).

The *SAC51* 5′-leader deletion restores the *acl5* phenotype

The mutation found in the *SAC51* uORF suggested that its effects were manifested through the altered translation of the main ORF encoding the bHLH protein. To examine whether deletion of the *SAC51* 5′-leader can also serve as a gain-of-function allele and restore the *acl5* phenotype, we made the *SAC51Δ5′* construct, which contains the *SAC51* coding sequence fused with its own promoter, but lacks the 5′-leader, and introduced it into *acl5-1*. We confirmed in all six independent transgenic lines that, although not completely,

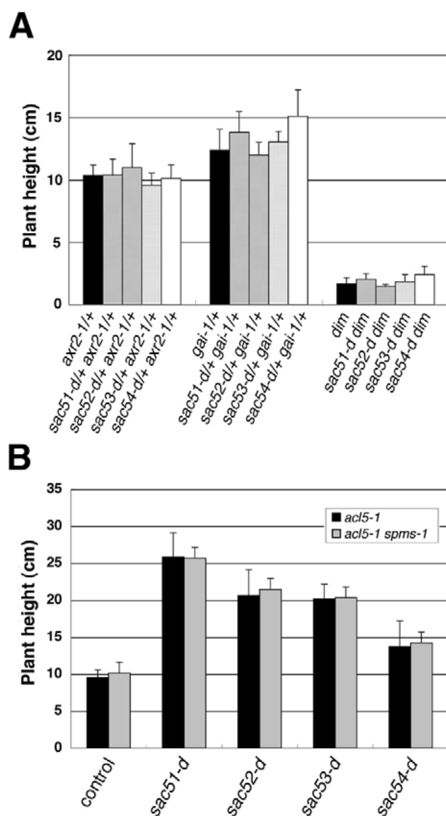

Fig. 4. Genetic interactions of *sac* with phytohormone-related and *spms* mutants. (**A**) Effect of each *sac* allele on the plant heights of 8-week-old *axr2*, *gai* and *dim* mutants. (**B**) Effect of each *sac* allele on the plant heights of 6-week-old *acl5* and *acl5 spms* mutants. Bars show mean±s.d. (*n*=6).

the construct restored the *acl5* dwarf phenotype. The *SAC51Δ5′* construct had no effect on the growth of wild-type plants. Moreover, overexpression of *SAC51* under the control of a heat-shock gene promoter in *acl5-1* also partially restored the phenotype in response to heat-shock treatments of the plants (see Fig. S2 in the supplementary material).

The *sac51-d* 5′-leader sequence increases the expression of the *GUS* fusion gene

We further generated chimeric gene constructs that consist of a 990-bp *SAC51* promoter fragment, the entire 5′-leader region of either the wild-type *SAC51* or *sac51-d* transcripts containing five uORFs and three introns, and the *GUS* reporter gene (Fig. 7A). These two constructs were introduced into wild-type plants and three independent transgenic lines that contained one copy of the transgene were obtained for each construct. In *SAC51-GUS* plants, GUS activity was detected in young leaves, roots, stems and flowers (Fig. 7B,C), with higher activity in the vascular tissues of both leaves and roots (Fig. 7D,E). In mature embryos, GUS activity was confined to the shoot apex and the root tip (Fig. 7F). In *sac51-d-GUS* plants, GUS staining was stronger in every tissue examined than in *SAC51-GUS* plants (Fig. 7B-F). The GUS activity was determined in five seedlings for each individual transformant and was normalized to the *GUS* transcript level to provide an indication of the translational efficiency of each construct. The *sac51-d-GUS* construct yielded 51.5-fold more GUS activity than the *SAC51-GUS* wild-type construct. In parallel with *sac51-d* transcript levels in *sac51-d acl5-1* plants (Fig. 6B), however, steady-state levels of the *GUS* transcript were markedly increased in *sac51-d-GUS* plants, when compared with those in *SAC51-GUS* plants. Thus, our results indicate that the *sac51-d* mutation causes a 2.7-fold increase in GUS translational efficiency, when compared with the wild-type *SAC51* construct (Fig. 7G).

To examine whether disrupting the peptide sequence of the *SAC51* uORF is crucial for suppression of the *acl5* phenotype, we generated another *GUS* construct carrying a C-to-A substitution at the site of the *sac51-d* mutation (*sac51-C549A-GUS*). This causes a Gln-to-Lys substitution of the fourth amino acid of the fourth uORF (Fig. 7H). This construct was introduced into wild-type plants and was found to have no obvious effect on transcription and translation of the *GUS* reporter gene, when compared with the wild-type *SAC51* construct (Fig. 7G). The results were reproduced in three independent transformants for each construct.

ACL5 may be involved in the translational activation of *SAC51*

To address possible regulatory interactions between *ACL5* and *SAC51*, we introduced both *SAC51-GUS* and *sac51-d-GUS* constructs into *acl5-1* and *sac51-d acl5-1* mutants by crossing experiments, and examined the *GUS* expression in these mutants. The GUS activity driven by the *SAC51-GUS* construct in *acl5-1* and *sac51-d acl5-1* seedlings was about 40% of the levels in the wild-type background, whereas the steady-state levels of the *GUS* transcript were unaffected in these mutant seedlings. Thus, the GUS translational efficiency in *acl5-1* and *sac51-d acl5-1* was estimated to be 38.8% and 48.2%, respectively, of the levels in the wild-type background (Fig. 7G). The steady-state levels of the *GUS* transcript from the *sac51-d-GUS* construct were markedly increased in *acl5-1* and *sac51-d acl5-1*, as in the wild-type background. The GUS activities in these mutants were consequently increased but reached to about 60% of the activities in the wild-type background. These results suggest that *ACL5* is required for full activation of *SAC51* translation.

The fact that *ACL5* encodes spermine synthase suggests a role of spermine in the translational activation of *SAC51*. A previous study reported that the *acl5* phenotype is restored by heat-shock treatments of *acl5-1* plants carrying the *HS-ACL5* construct (Hanzawa et al., 2000). Thus, we finally tested whether or not overexpression of another spermine synthase gene, *SPMS*, can rescue the *acl5* phenotype with the heat shock-inducible *HS-SPMS* construct. However, the results showed no effect of heat shocks on the growth of *acl5-1* plants carrying the *HS-SPMS* construct, suggesting that *ACL5* and *SPMS* take on different roles in the same cell (see Fig. S2B in the supplementary material).

DISCUSSION

sac mutants are specific suppressors of *acl5*

To elucidate the molecular pathways underlying the stem elongation mediated by the spermine synthase gene *ACL5*, we have isolated one dominant and three semi-dominant suppressor mutants of the *acl5* phenotype, designated *sac*. These *sac* mutants are not allelic to each other and suppress the *acl5* phenotype to different extents (Fig. 2A, Table 3). The *sac51-d acl5-1* mutant is nearly identical in appearance to the wild-type plant, whereas the *sac53-d acl5-1* mutant has differential effects in restoring the stem length and the stem diameter. The *sac54-d acl5-1* plant shows full recovery of the pedicel length but only partial recovery of the stem length. These findings suggest that *SAC53* and *SAC54* act in either

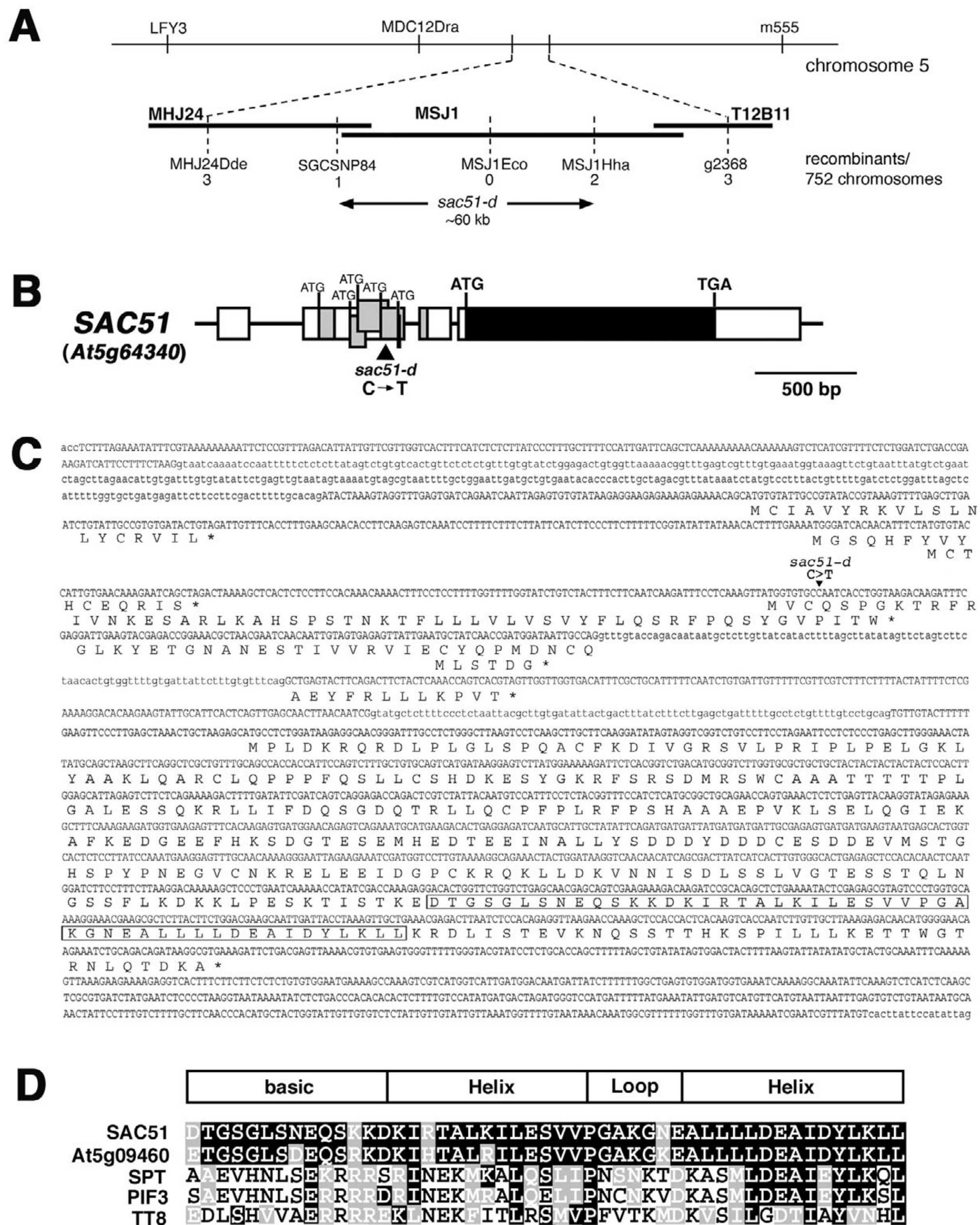

Fig. 5. Map-based cloning and sequence analysis of *SAC51*. (**A**) The region of chromosome 5 containing *SAC51*. The chromosome is depicted by the uppermost horizontal line with the franking markers LFY3 and m555. Below this are three P1 or BAC clones: MHJ24, MSJ1 and T12B11. The markers (see Materials and methods) and number of recombinants are shown. (**B**) Structure of the *SAC51* gene. Boxes indicate exons and the solid lines between boxes indicate introns. A black box represents a principal ORF and gray boxes represent uORFs. The location of the *sac51-d* mutation is shown. (**C**) The wild-type (Col-0) genomic DNA sequence of *SAC51* in which the regions corresponding to the cDNA shown in uppercase letters (GenBank Accession number: AY062561). The deduced amino acid sequences of the uORFs and main ORF are indicated below the nucleotide sequences. Asterisks indicate stop codons and the arrowhead indicates the position of the *sac51-d* mutation. The SAC51 bHLH domain is boxed. (**D**) Alignment of the bHLH domain of SAC51, its homolog At5g09460 and three characterized proteins from *Arabidopsis*: SPATULA (SPT) (Heisler et al., 2001), PHYTOCHROME INTERACTING FACTOR3 (PIF3) (Ni et al., 1998) and TRANSPARENT TESTA8 (TT8) (Nesi et al., 2000). Black blocks indicate residues identical to the SAC51 sequence; gray blocks indicate similar amino acids.

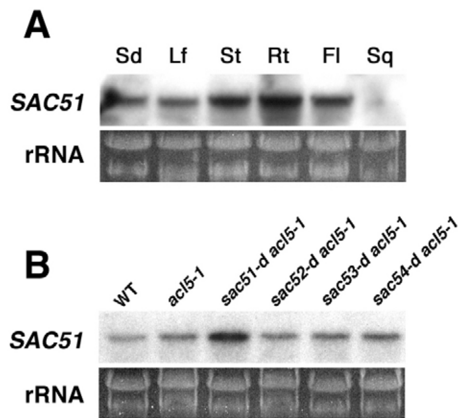

Fig. 6. Northern analysis of *SAC51* expression levels. (**A**) Tissue profiling of *SAC51* transcripts. Total RNA was prepared from 12-day-old seedlings (Sd), and from 6-week-old plant leaves (Lf), stems (St), roots (Rt), flowers (Fl) and green siliques (Sq). (**B**) *SAC51* transcript levels in wild-type (WT), *acl5,* and *sac acl5* plants. Total RNA was prepared from 12-day-old seedlings. Each lane contains 10 μg of total RNA and rRNA is shown as a loading control.

an organ- or tissue-specific manner in potential pathways downstream of functional *ACL5*. Because none of the four *sac* mutants suppress the dwarf phenotypes of the *axr2*, *gai* and *dim* mutants, we conclude that these *sac* alleles are not general suppressors of stem growth defects but specifically function in the *ACL5*-mediated growth regulatory pathways, which can be uncoupled from those of auxin, GA and BR.

Because of the presence of another spermine synthase gene in *Arabidopsis*, *SPMS*, all *sac acl5* mutants should still be able to produce spermine. This raised the possibility that the spermine produced by SPMS could function in the *ACL5*-mediated regulatory pathways in *sac* mutants. However, we found that all four *sac* mutants can also suppress the *acl5* phenotype in an *acl5 spms* background (Fig. 4B), indicating that the recovery of stem growth does not require spermine in these *sac* mutants. This result is consistent with the observation that, unlike the *HS-ACL5* construct, which can restore the *acl5* phenotype in a heat-shock-dependent manner (Hanzawa et al., 2000), the *HS-SPMS* construct had no effect on the growth of *acl5* plants carrying the construct (see Fig. S2B in the supplementary material). Determination of the subcellular localization of ACL5 and SPMS, and/or the identification of an ACL5-interacting protein, will be needed for further understanding of the functional difference between these two spermine synthases.

We observed that the transcript levels of the HD-Zip III class homeobox genes were increased in *acl5-1*, even at vegetative stages, prior to the visible manifestation of the *acl5* phenotype (Fig. 2D). These transcript levels were restored in each *sac acl5* mutant, and restoration correlated with the degree of recovery in plant height, suggesting a close association between HD-Zip III gene expression and reduced stem elongation. The *Arabidopsis* HD-Zip III class genes are mainly expressed in the procambium and developing vascular tissues (Baima et al., 1995; Zhong and Ye, 1999; Ohashi-Ito and Fukuda, 2003; Prigge et al., 2005). Overexpression of a member of this class, *ATHB8*, promotes xylem formation and results in a strong reduction in plant height (Baima et al., 2001). This may be a consequence of the accelerated differentiation of the primary vasculature, which in turn provokes the anticipated transition to secondary growth. Because *acl5-1* stem internodes also have abnormal vascular structures following the overproduction of xylem tissues, the defect in stem elongation in *acl5-1* might be attributable to the increased transcript levels of the HD-Zip III class genes, resulting in abnormal vascular differentiation.

A recent study of the *thickvein* (*tkv*) mutant, a new allele of *ACL5*, suggested that a defect in polar auxin transport is responsible for the *tkv* phenotype that is characterized by the abnormal vasculature in the leaves and stem internodes, and that *ACL5/TKV* functions in a mechanism that defines the boundaries between veins and the non-vein regions through correct polar auxin transport in provascular cells (Clay and Nelson, 2005). *ACL5* expression is upregulated by auxin and by the *acl5-1* mutation itself (Hanzawa et al., 2000). Moreover, the increased *acl5-1* transcript levels are restored in each of the *sac* mutants, in parallel with the suppression of the dwarf phenotype (Fig. 3). Taken together, these findings suggest that the defect in the polar auxin transport system in the *acl5-1* provascular cells causes a local increase in the auxin levels, which in turn results in the overproliferation of provascular cells and in the increased expression of the *acl5-1* and HD-Zip III genes. If this is indeed the case, the *sac* alleles identified would restore the polar auxin transport system in accordance with the recovery of vascular differentiation.

Posttranscriptional control of *SAC51* by its uORF

The *SAC51* transcript has a long 5′-leader containing five short ORFs upstream of its bHLH protein coding sequence (Fig. 5B). The *sac51-d* dominant allele was found to have a point mutation in one of these uORFs, which introduces a premature stop codon. Although uORFs occur relatively infrequently in eukaryotic mRNAs, their occurrence is more frequent in growth-related genes such as oncogenes (Kozak, 1987; Kozak, 1991). uORFs are known to impede the translational initiation of their downstream coding ORFs, and this translational repression is released under appropriate conditions (Morris and Geballe, 2000). For example, the yeast *GCN4* gene contains four uORFs. These allow the ribosome to reach the start codon of the main ORF only when cells are under amino acid starvation and need to express the GCN4 protein (Hinnebusch, 1990). Such uORF-mediated translational regulation has been investigated in several plant genes including *Opaque 2* (Lohmer et al., 1993) and *Lc* (Wang and Wessler, 1998) in maize, and *SAMDC* (Hanfrey et al., 2002) and *ATB2* (Wiese et al., 2004) in *Arabidopsis*. Our experiments with transgenic plants carrying *GUS* reporter fusion constructs revealed that the *sac51-d* sequence increases both the transcript levels and the efficiency of translation of the *GUS* reporter gene (Fig. 7G). We also confirmed that the stem growth of *acl5-1* was partially restored by transformation with the *SAC51* 5′-leader deletion construct (SACΔ5′) and the heat shock-inducible *SAC51* construct (HS-SAC51Δ5′) (see Fig. S2 in the supplementary material). Thus, we conclude that the *sac51-d* allele deregulates a posttranscriptional control of the *SAC51* gene and that the resulting overproduction of the SAC51 bHLH protein is responsible for the suppression of the *acl5* phenotype in *sac51-d acl5-1*. This is consistent with the dominant characteristics of *sac51-d*. A mutation similar to *sac51-d* has been also reported for a uORF of the *Arabidopsis ATR1* gene, which encodes a MYB transcription factor (Bender and Fink, 1998). The dominant *atr1D* allele was identified as an altered tryptophan regulation mutant, with increased expression of a target of ATR1, *ASA1*, which encodes the anthranilate synthase α subunit.

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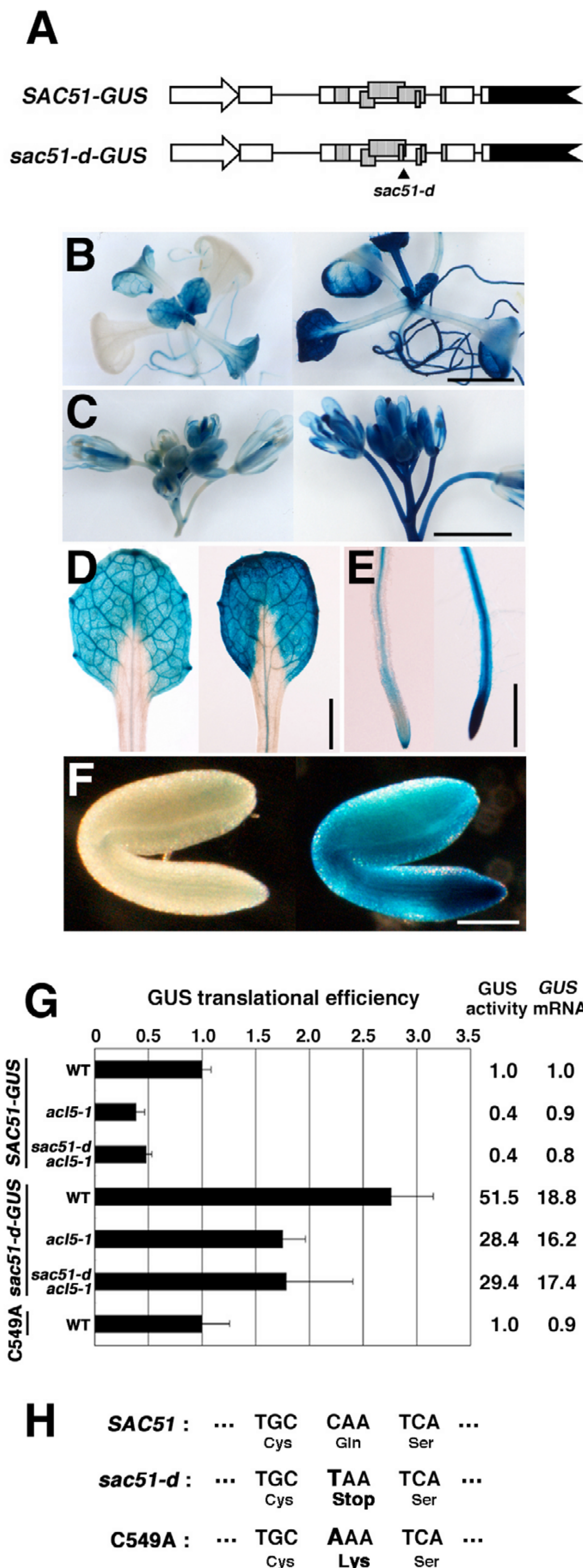

Fig. 7. Effects of the *SAC51* 5′-leader on *GUS* reporter gene expression. (**A**) The *GUS* gene constructs consisting of the *SAC51* promoter with either wild-type *SAC51* or the mutant *sac51-d* 5′-leader sequences and the *GUS* coding sequence. White arrows represent the *SAC51* promoter, boxes indicate exons, lines indicate introns; gray boxes represent uORFs and black blocks correspond to the *GUS*-coding sequence. (**B-F**) GUS staining (blue) in transgenic plants harboring the *SAC51-GUS* construct (left) or the *sac51-d-GUS* construct (right). Sixteen-day-old seedlings (B), inflorescences (C), young rosette leaves (D), roots (E), and mature embryos (F) are shown. Scale bars: B,C, 5 mm; D,E, 1 mm; F, 100 μm. (**G**) The relative GUS translational efficiency of *SAC51-GUS* and *sac51-d-GUS* constructs in wild-type (WT), *acl5-1* and *sac51-d acl5-1* backgrounds, and that of the *sac51-C549A-GUS (C549A)* construct in the wild-type background. The levels of GUS activity and *GUS* mRNA in the *SAC51-GUS* transgenic line in the wild-type background were set at 1.0. The GUS translational efficiencies were calculated as the GUS activity divided by the *GUS* mRNA level for each plant. The *GUS* mRNA levels were normalized to the *ACT8* level in each sample. Bars show mean±s.d. (*n*=3). (**H**) Nucleotide and amino acid changes at the *sac51-d* mutation site in *SAC51-GUS, sac51-d-GUS* and *sac51-C549A-GUS* constructs. Nucleotide and amino acid changes are in bold.

Why are the steady-state levels of both *sac51-d* and *sac51-d-GUS* transcripts much higher than those of *SAC51* and *SAC51-GUS* transcripts in respective plant lines (Fig. 6A, Fig. 7G)? The most likely possibility is that the *sac51-d* and *sac51-d-GUS* transcripts are stabilized by increased translation of their main ORF. Some studies with other plant systems have shown that the increased translation has the potential to protect a transcript from degradation (Sullivan and Green, 1993; Abler and Green, 1996). It is less likely that the *sac51-d* transcript is stabilized by the alteration its own secondary structure with the C-to-T substitution, because the C-to-A substitution at the site of the *sac51-d* mutation had no effect on the *GUS* expression.

Alternatively, the polypeptide encoded by the fourth uORF of *SAC51* might be involved in destabilizing its own mRNA. The uORF that contains a premature stop codon in *sac51-d* is the longest one of the five overlapping uORFs of the *SAC51* gene and is well conserved among all four members of the bHLH subfamily to which SAC51 belongs (Toledo-Ortiz et al., 2003). The presence of such a conserved uORF suggests that all of these bHLH proteins are under a common posttranscriptional control mechanism. However, we have so far identified no peptide sequences homologous to those of the *SAC51* uORFs in other plant species in the database. In *Neurospora crassa*, the *arg-2* gene encoding an arginine biosynthetic enzyme contains an evolutionarily conserved uORF. This uORF encodes the 24-amino-acid arginine attenuator peptide (AAP) that confers negative translational regulation in response to increased arginine levels (Luo and Sachs, 1996). At high arginine concentrations, AAP causes ribosomes to stall at the uORF termination codon and create a blockade of additional ribosome scanning. The peptide sequences encoded by uORFs are crucial regulators of some genes, including the cytomegalovirus *gpUL4* (Alderete et al., 1999) and the mammalian *SAMDC* (Raney et al., 2000), whereas those of the above mentioned *GCN4* gene are relatively unimportant for its function (Hinnebusch, 1996; Hinnebusch, 2000). A more detailed mutagenic analysis of the *SAC51* 5′-leader sequence will be needed to determine whether the function of the *SAC51* uORF is sequence-dependent or not.

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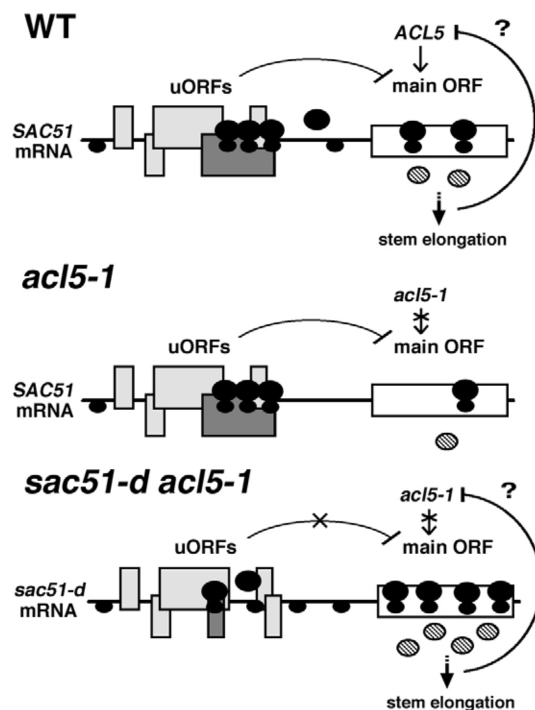

Fig. 8. A model of uORF-mediated translational control of *SAC51* expression via *ACL5* function. *SAC51* and *sac51-d* transcripts are represented as horizontal lines, the fourth uORF as a dark gray box, uORFs (except the fourth uORF) as light gray boxes, the main ORF as a white box and SAC51 bHLH proteins as hatched circles. Small (40S) and large (60S) ribosomal subunits are indicated by small and large black circles, respectively.

A model for *ACL5*-dependent translational regulation of *SAC51*

One important question is whether *ACL5* acts in the control of *SAC51* expression. A key finding of our experiments is that the translational efficiency of the *SAC51-GUS* construct was decreased in *acl5-1* and *sac51-d acl5-1* (Fig. 7G). This implies that the translational efficiency of the *SAC51* main ORF depends at least in part on the *ACL5* function. As polyamines are known to promote various stages of protein biosynthesis (Tabor and Tabor, 1984), it is possible that spermine is directly involved in the translational control of *SAC51*. In bacteria, spermidine facilitates the formation of the translation initiation complex (Yoshida et al., 1999). By contrast, in the mammalian *SAMDC* gene, polyamines may directly participate in an interaction between its uORF-encoded peptide and a constitutive component of the translation machinery, which leads to the inhibition of ribosome activity (Mize and Morris, 2001). We found that the GUS translational efficiency of the *sac51-d-GUS* gene in the wild-type background was decreased to about 60% in *acl5-1* and *sac51-d acl5-1* (Fig. 7G), indicating that *ACL5* enhances *SAC51* translation independently of the regulation by its uORFs. Taken together, we propose a model whereby *ACL5* acts, either directly or indirectly, as a translational activator of *SAC51*, and probably of its homologs (Fig. 8). In this model, we also hypothesize that the premature termination of a 53-amino-acid polypeptide encoded by the fourth uORF of *SAC51* facilitates the release of the ribosome from the uORF and the reinitiation of translation at the main ORF in *sac51-d*. The restoration of the *acl5-1* transcript level in *sac51-d acl5-1* plants, suggests that the negative-feedback control of *ACL5* expression does not require spermine itself. This feedback control might involve auxin homeostasis, as discussed above, or downstream components of either *SAC51* or its homologs. This model is supported by the histochemical localization of *SAC51-GUS* expression in transgenic plants (Fig. 7B-F), as *SAC51* shows high expression in the shoot apex and vascular tissues of young leaves and roots, a pattern similar to the expression domain of *ACL5* (Clay and Nelson, 2005).

We could not detect the SAC51 protein in plant extracts by immunoblotting. Attempts to fluorometrically and immunologically detect the SAC51-GFP fusion protein by expressing it under the control of the *SAC51* promoter and the *sac51-d* 5′-leader were also unsuccessful, although this fusion construct restored the *acl5* phenotype (data not shown). Because the SAC51 amino terminus contains sequences rich in proline, glutamic acid, serine and threonine (PEST), which are often found in rapidly degraded proteins (Rechsteiner and Rogers, 1996), SAC51 might be continuously accumulated and degraded below detectable levels.

In conclusion, our findings shed light on uORF-mediated posttranscriptional control in plant development for the first time. The identification of other *SAC* genes, and the downstream targets of *SAC51*, will further elucidate the exact roles of *ACL5* and *SAC51* during stem internode elongation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/18/3575/DC1

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