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ACAULIS5, an *Arabidopsis* gene required for stem elongation, encodes a spermine synthase

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Polyamines have been implicated in a wide range of biological processes, including growth and development in bacteria and animals, but their function in higher plants is unclear. Here we show that the *Arabidopsis ACAULIS5* (*ACL5*) gene, whose inactivation causes a defect in the elongation of stem internodes by reducing cell expansion, encodes a protein that shares sequence similarity with the polyamine biosynthetic enzymes spermidine synthase and spermine synthase. Expression of the recombinant ACL5 protein in *Escherichia coli* showed that *ACL5* possesses spermine synthase activity. Restoration of the *acl5* mutant phenotype by somatic reversion of a transposon-induced allele suggests a non-cell-autonomous function for the *ACL5* gene product. We also found that expression of the *ACL5* cDNA under the control of a heat shock gene promoter in *acl5* mutant plants restores the phenotype in a heat shock-dependent manner. The results of the experiments showed that polyamines play an essential role in promotion of internode elongation through cell expansion in *Arabidopsis*. We discuss the relationships to plant growth regulators such as auxin and gibberellins that have related functions.

Keywords: *ACAULIS5*/*Arabidopsis*/internode elongation/polyamines/spermine

Introduction

The external appearance of flowering plants is determined by temporal and spatial control of the formation of leaves, flowers and stem internodes. Following germination, the vegetative shoot meristem generates leaf primordia on its periphery. Upon receiving the appropriate developmental signals, it is transformed into an inflorescence meristem, which gives rise to flowers. The transition to the reproductive phase is often accompanied by drastic changes in the branching pattern and in the elongation growth of stem internodes (Weberling, 1989). In sunflowers (*Helianthus annuus*), for example, the shoot meristem produces leaves in unison with elongating internodes at the vegetative stage. After floral transition, it generates an inflorescence composed of hundreds of individual flowers, which is known as a head. In contrast, in the case of the model plant *Arabidopsis thaliana*, little internode elongation occurs during vegetative growth and a characteristic rosette is formed. On transition to reproductive development, flowers are formed on the flanks of the shoot, and rapid elongation of internodes occurs between the last 2–3 leaf nodes. Therefore, the mature flowering plant bears flowers on an elongated shoot above the rosette. On the other hand, the dandelion (*Taraxacum officinale*) is an example of a plant with rosette leaves, a single elongated internode and a head inflorescence. These varieties of shoot architecture suggest the existence of a developmental programme for internode elongation unique to individual species and also provide important criteria for their relationships.

Numerous studies based on identification and characterization of mutants with defects in internode elongation have revealed that some of these mutants accumulate very low levels of bioactive gibberellic acid (GA) and are defective in one of the steps of the GA biosynthetic pathway (for a review see Hedden and Kamiya, 1997). Recent genetic strategies using *Arabidopsis* mutants have led to the isolation of a series of genes involved in GA biosynthesis. Furthermore, isolation and analysis of suppressor mutants for GA-requiring dwarfs in *Arabidopsis* have led to the identification of transcription factors belonging to the plant-specific VHIID family (Peng *et al*., 1997; Silverstone *et al*., 1998). Of these suppressors, which are recessive and are thought to be loss-of-function mutations, one has been shown to be allelic to a GA-insensitive (*gai*) mutant, which represents a semi-dominant gain-of-function allele and shows a dwarf phenotype. It remains unclear, however, how their functions are inactivated by GA. Transcription factors of different classes have been identified from other GA-insensitive dwarf mutants, *tiny* (Wilson *et al*., 1996) and *short internode* (*shi*) (Fridborg *et al*., 1999). These two mutants also represent semi-dominant gain-of-function alleles because they were isolated by a transposon tagging strategy using a *Dissociation* (*Ds*) element with a cauliflower mosaic virus (CaMV) 35S promoter, which causes overexpression of the flanking plant gene (Wilson *et al*., 1996; Long *et al*., 1997). These findings suggest the presence of a regulatory mechanism that represses inappropriate elongation of stem internodes in *Arabidopsis*. Genes under the control of these transcription factors remain to be determined.

Because GA appears to participate in many aspects of plant development, including seed germination, flower induction and anther development, as well as internode elongation, it has been suggested that GA functions may be specified by its interaction with other phytohormones such as auxin and brassinosteroid and/or the presence of

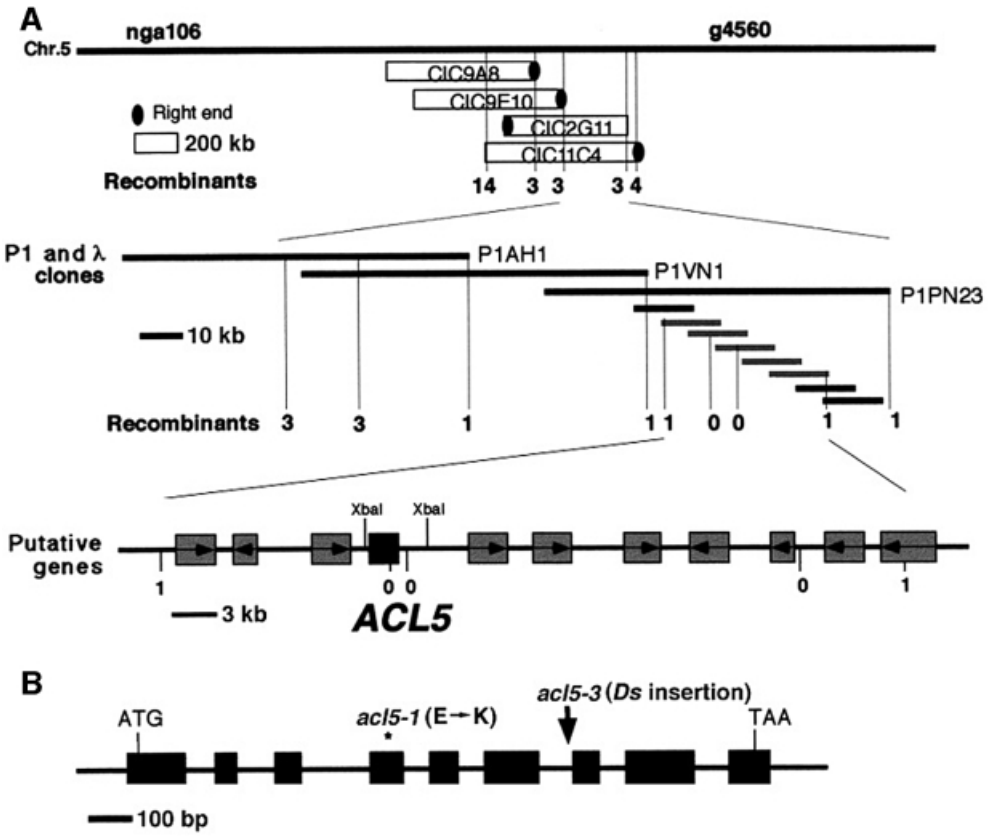

Fig. 1. Cloning and sequence analysis of the *ACL5* gene. (**A**) Summary of positional cloning of the *ACL5* gene. Molecular markers in the region and the number of recombinations between the marker and the *ACL5* locus are shown. Grey boxes represent putative ORFs around the *ACL5* locus (black box), whose direction of transcription is indicated by arrows. Restriction sites (*Xba*I) used for Ti-plasmid construction for complementation are indicated. (**B**) Genomic structure of the *ACL5* gene (AF184093, AF184094). The nine exons indicated by black boxes were determined by comparison between genomic and cDNA sequences. Positions mutated in *acl5-1* and *acl5-3* alleles are indicated.

downstream signalling molecules. However, we have little genetic information on such interactions and signalling cascades during the internode elongation. Furthermore, in order to unravel the cellular basis for elongation growth, it is also important to identify actual molecules that play a critical role in the process of cell elongation, which underlies the internode elongation. Nicol *et al*. (1998) identified one such gene, *KORRIGAN* (*KOR*), which encodes a membrane-anchored member of the endo-1,4-β-D-glucanase. The *kor* mutants are defective in organ elongation, suggesting a role for KOR in the correct assembly of the cellulose–hemicellulose network in the expanding cell wall.

To study further the control of shoot architecture, we have focused on *Arabidopsis* mutants named *acaulis5* (*acl5*). Recessive mutations in the *ACL5* gene result in a severe reduction in the length of stem internodes and cause a reduction in the number of flowers due to early proliferative arrest of apical inflorescence meristems (Hanzawa *et al*., 1997). The mutant plants show little or no morphological defects in other organs, unlike those defective in biosynthesis or perception of phytohormones, and the phenotype is not rescued by the addition of exogenous phytohormones. The transcript levels of the *GA5* gene encoding a GA 20-oxidase and other GA-related genes are affected in *acl5* plants, suggesting that ACL5 represents a novel regulator of the shoot development and functions downstream of GA response pathways. In the present study, we found that the *ACL5* gene encodes spermine synthase, one of the polyamine biosynthetic enzymes. Our results showing that introduction of the heat shock promoter–*ACL5* gene fusion into *acl5* plants leads to heat shock-dependent complementation of the phenotype may allow genetic manipulation of the shoot architecture, which is a long-standing goal in cultivated plants.

Results

Isolation of the ACL5 gene

To understand the molecular function of the *ACL5* gene product, we cloned the gene by positional cloning (Figure 1A). Genetic mapping experiments placed the *ACL5* locus between the right end of a yeast artificial chromosome (YAC) clone, CIC9F10, and the left end of a YAC clone, CIC2G11, on chromosome 5 (Schmidt *et al*., 1997). An *Arabidopsis* genomic library constructed in bacterial P1 plasmids was screened with these YAC end probes, and three positive clones were used to construct a λ phage library. Further genetic mapping experiments using restriction fragment length polymorphisms (RFLPs) delimited the *ACL5* locus to a 54 kb region covered by five overlapping phage clones derived from a P1 clone, PN23. The nucleotide sequence of this region was determined and computer analysis identified 11 putative

Fig. 2. Complementation of the *acl5-1* allele by the wild-type *ACL5* gene. Thirty-five-day-old plants of wild-type L*er*, *acl5-1* and transgenic *acl5-1* with the wild-type *ACL5* genomic fragment are shown from left to right. Transgenic *acl5-1* plants in the Col-0 background were backcrossed to the original *acl5-1* mutant in the L*er* background three times.

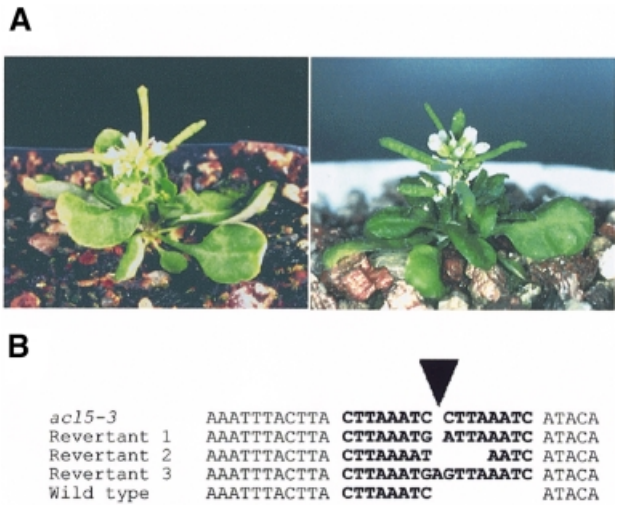

Fig. 3. *acl5* mutants caused by transposon insertion. (**A**) Phenotypes of *acl5-3* and *acl5-4* mutant alleles. The *acl5-3* allele (left) was caused by a *Ds* insertion into the *ACL5* gene. *acl5-4* (right) was identified as a deletion allele of the *ACL5* locus from the progeny of the cross between *acl5-3* and plants with an *Ac* transposase. (**B**) Sequences adjacent to the *Ds* element in the *acl5-3* allele and the footprints after excision of the *Ds* element in revertant alleles. The *Ds* element is represented as a triangle.

open reading frames (ORFs). Sequence determination of the ORFs of the mutant *acl5-1* identified a single base pair substitution in only one of these ORFs (Figure 1B). The *acl5-2* allele carried an identical mutation. Nine cDNAs that hybridized to this ORF were isolated, and they were all approximately the same length. Analysis of their sequence demonstrated that the gene consists of nine exons and eight introns. The mutant phenotype was fully corrected by introducing a 3.8 kb genomic fragment that contains a complete coding sequence of this gene into the *acl5-1* mutant (Figure 2).

ACL5 has a non-cell-autonomous function

In parallel, we identified another mutant allele, *acl5-3*, caused by a *Ds* transposon insertion (Figure 3A) (Long *et al*., 1993, 1997). The *Ds* element was inserted into the sixth intron of the same gene carrying the mutation in *acl5-1* (Figure 1B). Excision of the *Ds* element in the progeny of mutant plants by crossing with plants carrying the *Ac* transposase (TPase) gene caused one stable mutant allele (*acl5-4*) and revertant alleles that restored the wild-type phenotype (see Materials and methods). DNA fragments containing *Ds* excision sites in the revertant alleles were isolated by PCR, and the footprint sequences present after excision of *Ds* were determined (Figure 3B). Genomic DNA and RNA gel blot analyses revealed that the stable mutant allele, *acl5-4*, carried a large deletion of the locus and produced no detectable *ACL5* transcript (see below), indicating that it represents a null allele. Plants homozygous for the *acl5-4* allele showed the phenotype identical to that of *acl5-1* and *acl5-3* (Figure 3B).

In addition, in the F_2 generation from the cross between plants homozygous for *acl5-3* and those carrying a source of TPase, we obtained a remarkably low frequency of plants that carried the TPase construct and showed the *acl5* phenotype (only 3/613 plants, excluding the deletion of *acl5-4* allele, while a ratio of 3/16 would be expected by Mendelian segregation), and these mutant plants only produced wild-type progeny that also contained the TPase. This strongly suggests that the somatic excision of the *Ds* element from *acl5-3*, which is driven by TPase, corrects non-cell-autonomously the mutant phenotype of *acl5-3* to the wild-type phenotype so that mutants carrying TPase are exceedingly rare.

ACL5 encodes a polyamine biosynthetic gene

The predicted protein product encoded by the *ACL5* gene contains 339 amino acids and shows sequence similarity (32–36% identity) to spermidine synthase (EC 2.5.1.16) and 24–28% identity to spermine synthase (EC 2.5.1.22) from several organisms (Figure 4). Both of these enzymes have major roles in polyamine biosynthesis. Polyamines, low molecular weight polycationic molecules ubiquitously present in living organisms, have been implicated in a wide range of growth and developmental processes. The diamine putrescine is converted into spermidine and spermine through the consecutive activity of spermidine synthase and spermine synthase using decarboxylated *S*-adenosyl methionine (dcSAM) as an aminopropyl donor. BLAST searches indicated that both enzymes share 25–30% amino acid sequence identity with each other in animals and lower eukaryotes. While genes encoding spermidine synthase have been cloned from several plant species (Hashimoto *et al*., 1998; Alabadí *et al*., 1999), cloning of the plant spermine synthase gene has not been reported. The deduced protein sequence of the spermidine synthase gene of *A.thaliana* is 87% identical to a second putative spermidine synthase identified among expressed sequence tag clones (DDBJ/EMBL/GenBank accession No. AC005990) and 32% identical to ACL5. The amino acid substitution of glutamate at position 123 to lysine in the *acl5-1* allele occurs in a potential dcSAM-binding site (Posfai *et al*., 1989) and probably disables dcSAM binding (Figure 4). Southern hybridization with *ACL5* probes under low stringency conditions suggested that there is no gene

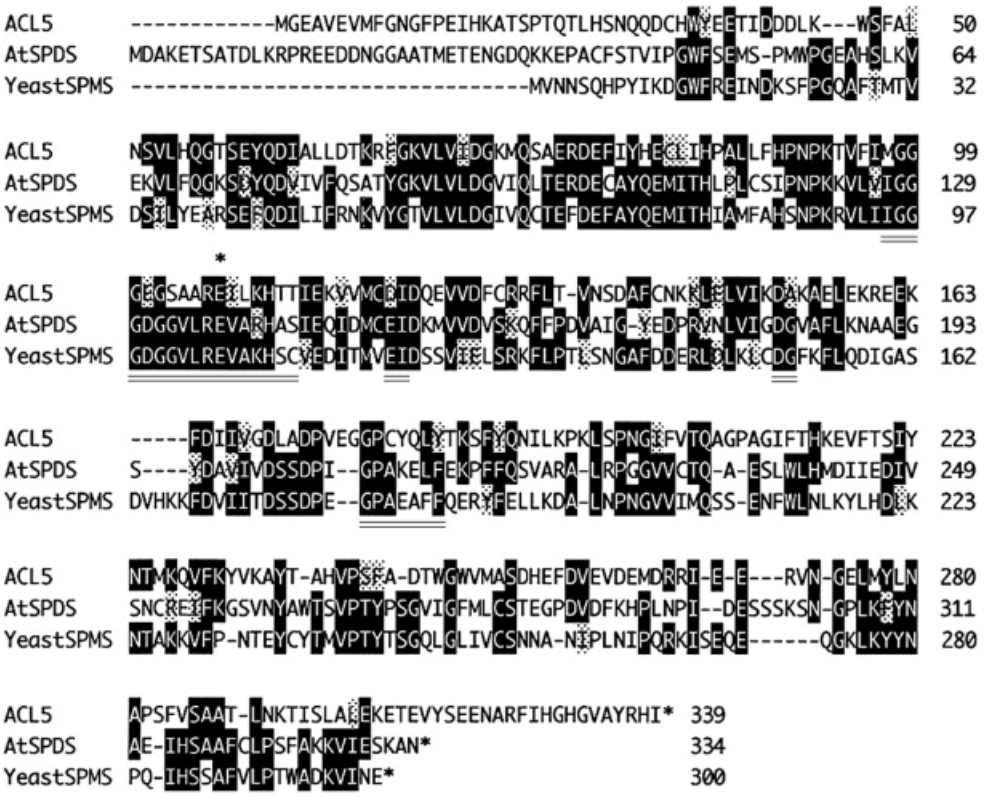

Fig. 4. Alignment of the deduced amino acid sequences of ACL5, *Arabidopsis* spermidine synthase (AtSPDS) (Hashimoto *et al*., 1998) and yeast (*Saccharomyces cerevisiae*) spermine synthase (Hamasaki-Katagiri *et al*., 1998). The potential decarboxylated *S*-adenosylmethionine-binding motifs (Posfai *et al*., 1989) are double underlined. Identities and similarities among the different proteins are indicated by black and grey boxes, respectively. The asterisk indicates the glutamate mutated in the *acl5-1* allele.

closely related to *ACL5* in the *Arabidopsis* genome (data not shown).

ACL5 possesses spermine synthase activity

In order to define the enzyme activity of the *ACL5* gene product, the wild-type ACL5 protein was produced in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP). High-performance liquid chromatography (HPLC) analysis of polyamines extracted from cells carrying the fusion construct revealed that ACL5 possessed spermine synthase activity, which is normally undetectable in *E.coli* cells (Figure 5). Conversion of radiolabelled spermidine into spermine in *E.coli* cells was also detected in feeding experiments (Table I). The mutated ACL5 was also produced by using a cDNA fragment derived from the *acl5-1* allele, and we confirmed that it had no spermine synthase activity (data not shown).

Expression analysis

RNA gel blot analysis showed that the *ACL5* transcript accumulated to high levels in stem internodes of adult flowering plants and also in root tissue (Figure 6A). This is in agreement with the effect of the mutation on elongation of the shoot and its slight effect on root elongation. Much lower levels of the transcript were observed in young seedlings before flowering and in rosette leaves, correlating with the lack of discernible phenotypes during vegetative growth of the mutant. Interestingly, *ACL5* transcript levels were much higher in the *acl5-1* mutant than in the wild-type, suggesting that *ACL5* expression may be under negative feedback control. The *ACL5* transcript could not be detected in the *acl5-4* allele carrying a large deletion of the locus (Figure 6B). Smeared intense signals in *acl5-3* may represent chimeric transcripts with the *Ds* sequence, truncated transcripts driven by the CaMV 35S promoter located in the *Ds* element (Long *et al*., 1997) and their degradation products.

The effects of phytohormones on polyamine metabolism have been implicated in some aspects of plant development. We therefore investigated whether *ACL5* transcript levels in wild-type seedlings are changed following hormone application. Auxin treatment with 100 μM indole-3-acetic acid (IAA) increased *ACL5* transcript levels (Figure 6C). Similar effects on *ACL5* expression were obtained after treatment with 1 μM IAA. Treatment with 100 μM benzyl aminopurine (BA), abscisic acid (ABA), brassinolid (BR) and gibberelic acid (GA3) had no influence on *ACL5* expression (Figure 6C). The upstream region of the *ACL5* gene appeared to contain putative auxin-responsive *cis*-acting elements (Figure 6D), which have been suggested to be a recognition site of transcription factors referred to as auxin response factors (Guilfoyle *et al*., 1998).

Manipulation of the internode length by transgenic ACL5 expression

To understand further the roles of the *ACL5* gene during the elongation of stem internodes, we generated transgenic

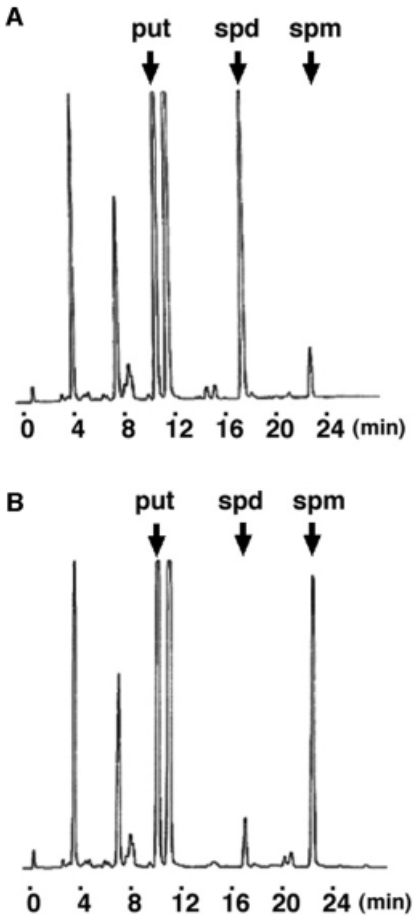

Fig. 5. ACL5 has spermine synthase activity. HPLC analysis of polyamine products in *E.coli* cells expressing ACL5. Extracts from cells carrying a pMAL empty vector (**A**) or the ACL5 fusion construct with MBP (**B**) were analysed by HPLC following the method of Flores and Galston (1982).

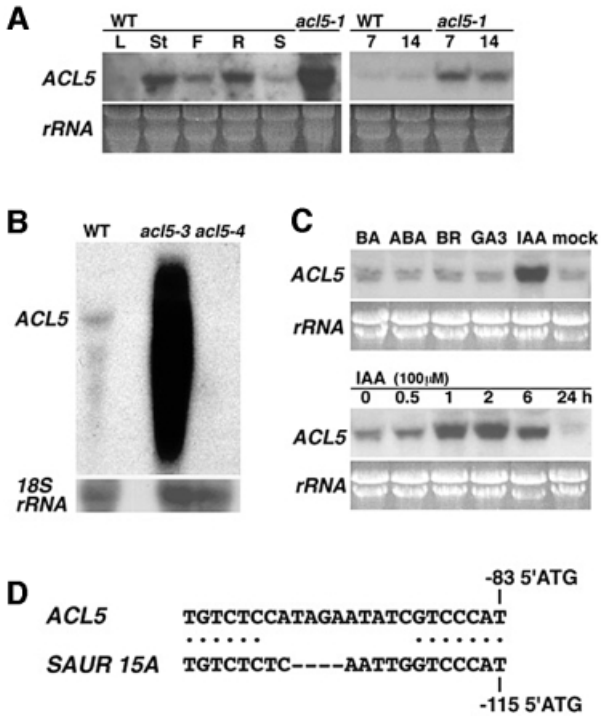

Fig. 6. *ACL5* expression patterns. (**A**) Organ-specific expression of *ACL5*. Northern blots were performed using 10 μg of total RNA per lane. Wild-type (WT) RNA samples in the left panels were prepared from leaves (L), stem internodes (St), flowers with apical meristems (F) and root tissue (R) of 35-day-old plants and 10-day-old seedlings (S). RNA of *acl5-1* was prepared from whole tissue of 35-day-old mutant plants. RNA samples in the right panels were prepared from 7- and 14-day-old seedlings of wild-type and *acl5-1* plants. (**B**) Expression of *ACL5* in *acl5-3* and *acl5-4* alleles. RNA samples were prepared from primary shoots of flowering plants. (**C**) Phytohormone-induced expression of *ACL5*. RNA samples were prepared from wild-type seedlings grown for 10 days in liquid culture media and treated with 100 μM each of phytohormones for 2 h (upper panels) or with 100 μM IAA for the indicated periods (lower panels). Northern blots were performed using 10 μg of total RNA per lane. (**D**) Putative auxin-responsive *cis*-elements (AuxREs) in the *ACL5* promoter region. The upstream sequence of the *ACL5* gene is compared with that of the soybean *SAUR 15A* gene (Li *et al*., 1994). Two putative AuxREs (TGTCTC and GTCCCAT) (Guilfoyle *et al*., 1998) are indicated by dots.

acl5-1 plants carrying the full-length *ACL5* cDNA fused with a heat shock-inducible promoter, and we examined the effect of heat shock-dependent overexpression of the *ACL5* gene on various stages of plant development. Plants homozygous for *acl5-1* (Col-0 background) were transformed directly with a T-DNA construct containing the *Arabidopsis HSP18.2* gene promoter–*ACL5* cDNA fusion gene. When young seedlings of transgenic *acl5-1* lines were treated with heat shock, they showed no inducible phenotype and were indistinguishable from non-treated mutant plants. The treatment did not affect the mutant phenotype after flowering. When treated with heat shock after flowering, however, these transgenic plants dramatically restored the phenotype (Figure 7A). We detected a tight correlation between the frequency of heat shock treatments and the degree of restoration in the length of stem internodes.

We also introduced a T-DNA construct for antisense expression of the *ACL5* cDNA under the control of the CaMV 35S promoter into wild-type plants and obtained five transformants. In one of the transgenic lines, #5, all of the progeny plants that were resistant to kanamycin exhibited a reduction in the length of stem internodes (Figure 7B). Although less frequently, the other three lines also showed a similar semi-dwarf phenotype in their progeny plants. Northern blot analysis revealed that these plants expressed a greatly reduced level of the *ACL5* transcript (data not shown).

Discussion

Identification of a novel component of shoot development

Recessive mutations in the *Arabidopsis ACL5* gene result in plants showing a severe defect that is restricted to cell elongation after transition to the reproductive stage. The results of our previous study suggested that the wild-type *ACL5* gene product represents a novel and essential regulator of the inflorescence development in flowering plants (Hanzawa *et al*., 1997). In the present study, we isolated the *ACL5* gene by positional cloning and revealed that it encodes a polyamine biosynthetic enzyme. Polyamines, a class of aliphatic amines, have been implicated in a wide range of growth and developmental

Table I. Radioactivities of the fractions corresponding to putrescine, spermidine and spermine in *E.coli* extracts

Plasmid	Putrescine (d.p.m.)	Spermidine (d.p.m.)	Spermine (d.p.m.)
pMAL	49	2753	31
	52	3215	83
pMAL/ACL5	56	469	1824
	45	352	1810

Feeding experiments with [^{14}C]spermidine were performed twice for *E.coli* cells carrying each construct (see Materials and methods).

Fig. 7. Transgenic expression of the *ACL5* cDNA. (**A**) Heat shock-dependent complementation of the phenotype in transgenic *acl5-1* plants. The full-length *ACL5* cDNA was cloned into a binary vector, pTT101 (Matsuhara *et al*., 2000), which contains the heat shock-inducible *Arabidopsis HSP18.2* gene promoter, and it was introduced into the *acl5-1* mutant. Plants were heat shocked at 37°C for 30 min every third day after flowering. The frequencies of heat shock treatment are indicated. (**B**) Phenotypes caused by antisense *ACL5* expression. The T-DNA construct for antisense expression of the full-length *ACL5* cDNA under the control of the CaMV 35S promoter was introduced into wild-type (Col-0) plants. Wild-type plants (WT) and the T2 progeny plants from transgenic lines #1 and #5 are shown.

processes in bacteria, animals and plants. Although it is well known that polyamines are involved in cell proliferation of both prokaryotes and eukaryotes, their exact role has yet to be firmly established. In animals, the gene encoding ornithine decarboxylase (EC 4.1.1.17), by which ornithine is converted to putrescine, has been suggested to be a proto-oncogene (Auvinen *et al*., 1992). In addition to the involvement of polyamines in DNA, RNA and protein synthesis, specific interactions with certain potassium channels and glutamate receptors have been reported (Ficker *et al*., 1994; Fakler *et al*., 1995; Williams, 1997). In higher plants, increases in polyamine biosynthetic activities have been observed in a wide range of developmental processes (Evans and Malmberg, 1989). Tobacco mutants resistant to the inhibitor of SAM decarboxylase (EC 4.1.1.50) exhibit abnormal patterns of floral organ development (Malmberg and McIndoo, 1983). The analysis of transgenic plants with engineered sense and antisense genes for polyamine biosynthetic enzymes indicates that changes in internal polyamine levels can affect stem elongation, leaf morphology and root growth (Kumar *et al*., 1996; Masgrau *et al*., 1997). However, there have been no definitive conclusions on the physiological role of polyamines in plant growth and development because of the lack of mutants that are defective in a single polyamine biosynthetic gene (Watson *et al*., 1998).

The high similarity between ACL5 protein and spermidine synthase or spermine synthase from other organisms suggests that ACL5 functions as a key enzyme in the polyamine biosynthesis pathway in plant cells. This suggestion is supported by results from expression of the *ACL5* cDNA in *E.coli* cells. ACL5 protein catalyses the pathway that converts spermidine into spermine, indicating that ACL5 can act as a spermine synthase in plants. This result is consistent with the fact that the exogenous addition of DL-α-difluoromethylornithine (DFMO) to wild-type plants causes a severe defect in the internode elongation, which is similar to the *acl5* mutant phenotype (data not shown). DFMO is an inhibitor of ornithine decarboxylase and is thought to reduce the endogenous level of putrescine, a precursor of spermidine and spermine. However, the possibility that ACL5 exhibits broad amine substrate specificities and may also be involved in the synthesis of other polyamines in plant tissues cannot be excluded. Although many uncommon polyamines, which differ from the common aliphatic polyamines in the number of methylenic moieties between the amine groups, have been discovered, especially in plants and thermophilic bacteria, their biosynthetic enzymes remain largely unidentified (Phillips and Kuehn, 1991).

Sequence analysis of the *acl5-1* allele revealed that the mutation caused amino acid substitution (E to K) in a

highly conserved residue within a potential dcSAM-binding site. When expressed in *E.coli*, the mutated ACL5 protein derived from the *acl5-1* allele had no spermine synthase activity. Taken together with the fact that the phenotype of *acl5-1* plants is identical to that of the deletion allele, *acl5-4*, it is likely that the *acl5-1* allele represents a complete loss-of-function mutation. Further biochemical analyses of recombinant ACL5 proteins by site-directed mutagenesis are currently underway and should help to elucidate specific amino acids necessary for spermine synthase activity.

Control of internode elongation by ACL5

Isolation of the *ACL5* gene provides the first genetic evidence confirming that polyamines play an important role in plant development. Although expression of the *ACL5* gene occurs mainly in those tissues in which the mutation has its greatest effect, it is also likely that ACL5 can act non-cell-autonomously like phytohormones to regulate shoot elongation. This is suggested by the results of experiments in which plants homozygous for *acl5-3*, the allele induced by insertion of the *Ds* element, were crossed to those carrying a source of the TPase. The extremely low frequency of mutant plants carrying the TPase in the F_2 families strongly suggests that somatic excision of *Ds* from *acl5-3* is sufficient to restore the phenotype and that polyamines produced by ACL5 can be transported. This is in good agreement with recent biochemical and physiological data suggesting that polyamines can be transported over distances through xylem and phloem (Caffaro *et al*., 1993; Antognoni *et al*., 1998). In animal systems, it has been shown that polyamines are moved via specific transporters and that the transport is energy dependent (Seiler and Dezeure, 1990). Regardless of whether or not polyamines can be defined as phytohormones, identification of such transport systems is expected to provide new insights into the actions of polyamines in plant cells. Although less likely, the possibility that the mutant phenotype is rescued by the movement of the ACL5 protein itself from cell to cell in *acl5-3* mutants carrying the TPase cannot be ruled out.

Application of exogenous spermine failed to rescue the *acl5* mutant phenotype in our feeding experiments (data not shown). One possible explanation for this is that additional polyamine products may be produced by ACL5 in wild-type *Arabidopsis* tissue and, therefore, that spermine alone cannot restore normal growth. Alternatively, exogenous spermine might not be transported to its proper intracellular targets in a physiologically active form. This is partly because of the existence of the cell wall, in which interactions of spermine with negatively charged pectic substances can block its uptake by cells (Bagni and Pistocchi, 1991). Polyamine-oxidizing enzymes tightly bound to the cell wall (Slocum, 1991) might also eliminate supplied spermine. As a further complication, plant polyamines exist predominantly in conjugated forms with hydroxycinnamic acids (Flores and Martin-Tanguy, 1991). Even if such conjugates are physiologically active, it remains unknown whether or not exogenous spermine can be integrated into their biosynthetic pathways.

RNA gel blot analysis revealed that expression of the *ACL5* gene is up-regulated by auxin. In addition, we identified a putative auxin-responsive *cis*-acting element in the upstream region of the *ACL5* gene. Considering the critical requirement for auxin in plant cell division and expansion, it is possible that early proliferative arrest of apical inflorescence meristems and/or reduced cell length in stem internodes in *acl5* mutants are caused by a defect in an ACL5-mediated response to auxin. Alternatively, we have shown previously that the *acl5* mutation affects expression levels of GA-regulated genes in stem internodes (Hanzawa *et al*., 1997), suggesting ACL5 involvement in GA signal transduction or GA biosynthesis. These data raise the possibility that interactions of auxin and GA in shoot development, on which there have been numerous reports, may be mediated in part by polyamines. Further study with phytohormone-related mutants should help to clarify the relationships between auxin, GA and polyamines. It would be interesting to examine whether the morphological phenotypes of such mutants can be partially restored by *ACL5* overexpression.

Our observations showing that *acl5-1* mutants have increased levels of *ACL5* transcripts compared with that of the wild-type suggest the presence of a negative feedback mechanism for *ACL5* expression. Similar observations have been reported for genes involved in GA biosynthesis (Phillips *et al*., 1995). It remains to be determined whether the *acl5* mutation also affects expression of other genes in polyamine biosynthetic pathways. To date, there has been little information on expression profiles of genes related to polyamine biosynthesis and those responsive to polyamines. Experiments to address this issue might be possible by using transgenic *acl5-1* plants carrying a heat shock-inducible *ACL5* cDNA. It should be noted that the *acl5* phenotype is restored dramatically by heat shock treatments of these transgenic plants. Taking into account that heat shock treatments of young seedlings have no effect and that repeated heat shocks of flowering plants are more effective for the restoration of the phenotype than a single heat shock pulse, it is thought that the internode elongation may be triggered by rapid and transient action of the *ACL5* gene product in transgenic mutant plants. Finally, this approach using an inducible promoter will not only provide a new opportunity to determine the precise mode of action of polyamines during internode elongation but also open the way for manipulation of the shoot architecture of flowering plants by genetic engineering.

Materials and methods

Plant material and growth conditions

Isolation of the *acl5-1* and *acl5-2* mutant alleles in the Landsberg *erecta* (L*er*) ecotype has been described previously (Hanzawa *et al*., 1997). Sequence determination of the *acl5-2* allele (see below) revealed that it contained the same mutation as the *acl5-1* allele, and it is therefore likely that they were not independently induced mutations. Another ecotype, Columbia (Col-0), was used as the wild-type for genetic experiments. Plants were grown in a growth chamber at 22°C under continuous fluorescent light. For RNA preparation from wild-type seedlings treated with phytohormones, seeds were surface sterilized, and sown and grown in an aerated solution of MS salts with 2% sucrose. The *acl5-3* allele was identified in a transposon-tagged mutant screen by using a two-component *Activator/Dissociation* (*Ac*/*Ds*) strategy described previously (Long *et al*., 1993, 1997). The stable deletion allele of *acl5* (*acl5-4*) was identified among the F_2 population from the cross between the *acl5-3* plants and plants carrying the CaMV 35S promoter::TPase gene construct. Eight F_1 plants were self-fertilized, generating eight separate F_2 families. The segregation ratio of phenotypically wild-type plants to

acl5 mutant plants was nearly 15:1 (the combined ratio in all families was 613 wild-type:32 mutants). Of the 32 mutants, only four carried the TPase construct, which can easily be determined because 35S::TPase also carries a bacterial β-glucuronidase (GUS) gene (Long *et al*., 1993, 1997). One of these four plants was shown to carry a stable deletion allele of *acl5* (*acl5-4*), which gave rise to a mutant phenotype in the presence of the TPase, by Southern blot hybridization. For the Southern hybridization, wild-type and *acl5-4* DNA were cleaved with *Xba*I and hybridized to the *ACL5* cDNA, which detected a strongly hybridizing fragment of 3.8 kb in the wild-type and a weakly hybridizing fragment of 1.4 kb in *acl5-4* (data not shown). Revertant alleles from *acl5-3* were isolated by crossing the *acl5-3* plants with plants homozygous for both the 35S::TPase construct and the *acl5-4* allele. In the F_2 progeny with the wild-type phenotype, three plants that did not carry the TPase gene construct were identified.

Cloning and sequencing of ACL5

Genetic crosses were performed as described previously (Hanzawa *et al*., 1997). DNAs from F_2 *acl5-1* homozygous plants were prepared for simple sequence length polymorphisms (SSLPs) (Bell and Ecker, 1994) and cleaved–amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993) analysis. F_2 *acl5-1* homozygous plants with recombination break points in the nga106-*ACL5* region (28 recombinants) or in the *ACL5*-ttg region (180 recombinants) were used to map SSLP, CAPS and RFLP molecular markers relative to the *ACL5* locus by Southern blot hybridization or PCR. For further fine mapping, the ends of YAC clones CIC9A8, CIC9F11, CIC2G11 and CIC11C4 were amplified by tail-PCR and used as RFLP markers. The right end of CIC9F10 and the left end of CIC2G11 were used as probes to isolate P1 clones (Research Institute of Innovative Research for the Earth and Mitsui Plant Biotechnology Research Institute). A genomic library was then constructed in λ-EMBL3 (Stratagene) phages by using restriction fragments of the P1 clone, PN23. Contiguous phage clones were used to identify RFLPs between two ecotypes and delimit the *ACL5* locus to a 54 kb region. DNASIS version 2.0 (Hitachi Software) was used to identify ORFs. All of the putative ORFs were PCR amplified and sequenced from the *acl5-1* allele with gene-specific primers. A labelled DNA fragment containing the *ACL5* gene was used as a probe to screen an *Arabidopsis* cDNA library constructed in λ ZAPII (Clontech). Protein databases were searched using the BLAST program.

For isolating plant DNA flanking a *Ds* insertion from the *acl5-3* allele, inverse PCR was performed as described previously (Wilson *et al*., 1996).

Plant transformation

For complementation, the bacterial GUS gene in the binary Ti-vector, pBI101 (Clontech), was replaced with a 3.8 kb *Xba*I genomic fragment containing a complete coding sequence of the *ACL5* gene (Figure 1A). For heat shock-inducible *ACL5* expression, a *Bam*HI–*Sal*I fragment of the full-length *ACL5* cDNA was cloned into the heat shock cassette Ti-vector pTT101 (Matsuhara *et al*., 2000). The CaMV 35S promoter::antisense *ACL5* construct was made by replacing the GUS gene in pBI121 (Clontech) with a *Sac*I–*Xba*I fragment of the full-length *ACL5* cDNA. The resulting constructs were introduced into *Agrobacterium* C58C1 strain and used to transform homozygous *acl5-1* or wild-type plants in the Col-0 background by the vacuum infiltration method (Bechtold *et al*., 1993). Transgenic seedlings were selected by resistance to kanamycin and transplanted to soil.

Expression in E.coli

The PCR fragment of the wild-type *ACL5* cDNA was cloned into the pMAL-c2 vector (New England Biolabs). The resulting construct was introduced into *E.coli* TB1 strain. The MBP–ACL5 fusion protein was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to culture media for 4 h. Polyamines were extracted according to the method of Flores and Galston (1982). In brief, cells in 2.0 ml of culture medium were harvested and homogenized with 0.4 ml of trichloracetic acid (5% w/w) on ice. The homogenate was centrifuged (10 000 *g*, 10 min), and 0.2 ml of the supernatant was mixed with 0.2 ml of saturated Na_2CO_3 and 0.4 ml of dansyl chloride (5 mg/ml acetone) and incubated in darkness overnight. The fluorescent polyamines extracted were analysed by HPLC. For feeding experiments, 10 µl of [^{14}C]spermidine (4.14 Gbp/mmol) was added to the culture media with IPTG. The fractions corresponding to putrescine, spermidine and spermine were collected and counted using a scintillation counter.

RNA gel blot analysis

Total RNA was prepared by the SDS–phenol extraction method. For northern hybridization, 10 µg of total RNA was separated on formaldehyde-denatured agarose gels, blotted onto GeneScreen nylon membranes (New England Nuclear) and hybridized to ^{32}P-labelled *ACL5* cDNA probes. Hybridizations and washes were performed according to the manufacturer's instructions.

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