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The *Lateral suppressor (Ls)* gene of tomato encodes a new member of the VHIID protein family

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ABSTRACT **The ability of the shoot apical meristem to multiply and distribute its meristematic potential through the formation of axillary meristems is essential for the diversity of forms and growth habits of higher plants. In the *lateral suppressor* mutant of tomato the initiation of axillary meristems is prevented, thus offering the unique opportunity to study the molecular mechanisms underlying this important function of the shoot apical meristem. We report here the isolation of the *Lateral suppressor* gene by positional cloning and show that the mutant phenotype is caused by a complete loss of function of a new member of the VHIID family of plant regulatory proteins.**

The pattern of shoot branching and the growth characteristics of lateral shoots determine to a large extent the growth habit of plants. In seed plants, shoot branching is initiated at the shoot apex with the formation of axillary meristems. In the axils of developing leaf primordia, distinct groups of meristematic cells, which are in direct continuity with the shoot apical meristem, can be identified by histological means, because of their dense cytoplasm and the low degree of vacuolation (1). This cell group proliferates and forms a dome-shaped axillary meristem, whose structure is very similar to that of the apical meristem of the primary shoot. After the formation of the first leaf primordia, development of these lateral buds often pauses due to the inhibitory effect of the shoot apex of the main shoot (2). In some plant species, the apical meristem of the primary shoot remains active throughout the life of the plant and continues to initiate the formation of lateral organs. In other plant species, the primary apical meristem at some point of development undergoes the transition to floral development or it aborts. In these cases, development is continued by one or few axillary meristems forming a sympodial shoot (e.g., *Lycopersicon esculentum* and *Petunia hybrida*).

Little is known about the genetic control of shoot branching. Mutants that exhibit either a reduced or an enhanced outgrowth of axillary buds have been described in various plant species (3). In other cases, the initiation of axillary meristems is blocked in some or most of the leaf axils. The *lateral suppressor (ls)* mutant of tomato is characterized by phenotypic abnormalities at different stages of development. During vegetative development the cells in the axils of leaf primordia fail to retain their meristematic character leading to the absence of side-shoots (4). However, at the transition to reproductive development, axillary meristems are initiated in the two leaf axils preceding the inflorescence. Whereas the uppermost axillary meristem develops into a sympodial shoot continuing the main axis of the plant, the second axillary meristem will develop into a side-shoot (4). Inflorescence development of homozygous *ls* plants is characterized by a lower number of flowers per inflorescence, the absence of petals (5), and a reduction in male and female fertility (6). The morphological defects of the *ls* mutant are accompanied by drastic changes in the levels of several plant hormones. In comparison to wild type, apices of homozygous *ls* plants contain much higher levels of auxins and gibberellic acid, whereas the levels of cytokinins are reduced (7). By studying the molecular processes underlying this complex phenotype, we hope to gain insight into important aspects of plant development. Based on previously reported genetic and physical mapping (8), we have isolated the *ls* gene by positional cloning. Sequence analysis of the mutant alleles reveals that the *ls* phenotype is caused by a complete loss of function of a member of the newly emerging family of VHIID regulatory proteins.

MATERIALS AND METHODS

Plant Materials. Tomato seed material of *L. esculentum* cv. Antimold B, Antimold B-*ls*[1] and Moneymaker was obtained from the Tomato Genetics Stock Center, Davis, CA. Tomato seeds of *L. esculentum* cv. Primabel and Primabel-*ls*[2] were obtained from J. Philouze (Institut National de la Recherche Agronomique, Montfavet, France). Plants were grown under standard glasshouse conditions with additional artificial light (16-h photoperiod) during the winter period.

DNA Isolation and Southern Blot Analysis. Plant DNA for PCR and Southern blot analysis was prepared as described (9). For Southern blot analysis, approximately 5 μg of genomic DNA was subjected to electrophoresis through 0.8% agarose, blotted to HybondN$^+$ membranes (Amersham Buchler, Braunschweig, Germany) and hybridized with radiolabeled probes. All standard techniques were carried out according to Sambrook *et al.* (10), unless otherwise stated.

RNA Isolation and Reverse Transcription (RT)-PCR Analysis. Total RNA was isolated by using the RNeasy system (Qiagen, Hilden, Germany) following the manufacturer's instructions. For RT-PCR analysis, 1 μg of total RNA was digested with DNaseI and reverse-transcribed by using the Superscript II polymerase (Life Technologies, Eggenstein, Germany) according to the manufacturer's instructions. The product of the first-strand cDNA synthesis reaction was amplified by PCR using the *Ls*-specific primers CD61–6 (5′-GGTGGCAATGTAGCTTCCAG-3′) and CD61–23 (5′-CCAGCTATTCAAATACGCCAG-3′). Amplification of ac-

Abbreviations: *ls, lateral suppressor;* RT, reverse transcription; RACE, rapid amplification of cDNA ends; YAC, yeast artificial chromosome; SCR, Scarecrow; GAI, Gibberellin insensitive; RGA, repressor of ga1–3.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF098674).

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tin cDNA by using primers specific for the potato gene PoAc101 (11) was performed as a control to ensure that equal amounts of cDNA were added to each PCR.

cDNA Isolation and Rapid Amplification of cDNA Ends (RACE) Experiments. A tomato (cv. VFNT Cherry) shoot tip cDNA library (12) was screened by using either yeast artificial chromosome (YAC) 61–5, the whole cosmid contig (Fig. 1*A*) or the insert of cosmid G as a probe. In each experiment at least 1×10^6 plaques were screened.

For RACE experiments (13), first-strand cDNA synthesis was performed as described above for RT-PCR analysis. RACE experiments were performed by using the RACE system of Life Technologies according to the manufacturer's instructions. For 5′ RACE, the standard adapter primer BRL-UAP and the *Ls*-specific primers CD61–30 (5′-TGATGGACTAACCGTTCAG-3′), CD61–11 (5′-AGCTAATGAGTAGCTGGCGG-3′), and CD61–31 (5′-TTGGAGTTGTTTCAACAGG-3′) were used. Amplified fragments were cloned into the pGEM-T (Promega) plasmid vector.

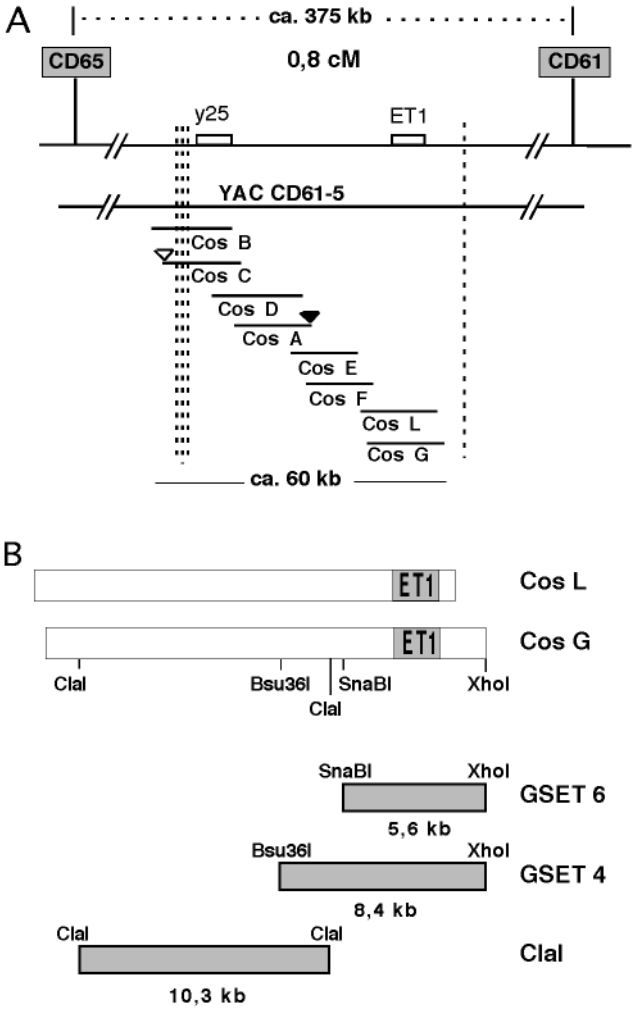

FIG. 1. Physical map of the *Ls* region. (*A*) Schematic representation of the cosmid contig around the *Ls* locus. The YAC and cosmid clones are shown as horizontal lines. The dashed vertical lines indicate the approximate positions of recombination breakpoints. Open boxes represent cDNAs, and flanking restriction fragment length polymorphism markers are displayed as shaded boxes. Cosmid end probes used for orientation of the cosmid contig are indicated as an open (C-for) or closed triangle (A-rev). (*B*) DNA fragments used for complementation experiments. The open bars represent the cosmid clones G and L. Shaded bars indicate different DNA fragments of cosmid G used for complementation. The position of the ET1 cDNA is displayed as a shaded box.

Isolation of YAC Clones. The PCR primers CD61-F, CD61-R, CD65-F, and CD65-R deduced from the restriction fragment length polymorphism markers CD61 and CD65, respectively (8), were used to screen a pooled tomato YAC library (14). To identify a YAC clone spanning the CD61-CD65 interval (8), CD61-positive clones also were analyzed with CD65 primers, and vice versa. After separation of agarose-embedded yeast chromosomes by pulsed field gel electrophoresis, YAC DNA was extracted by Gelase (Biozym, Hess. Oldendorf, Germany) digestion, radioactively labeled by using standard procedures, and used to screen a cDNA library.

Construction of a Binary Vector Cosmid Library. Genomic DNA of *L. esculentum* cv. Moneymaker was partially digested with *Mbo*I, and a 17- to 23-kb size fraction was ligated into the *Bam*HI site of the binary cosmid vector pCLD04541 (15), packaged by using commercial extracts (Gigapack II, Stratagene), and transfected into the *Escherichia coli* strain SURE (Stratagene). Approximately 250,000 recombinant clones with an average insert size of 20 kb representing more than five genome equivalents were divided into 100 pools. Screening of the library was done by PCR analysis of DNA from each pool followed by conventional colony filter hybridization.

Tomato Transformation. DNA fragments were cloned into the binary vectors pCLD04541 (15) or pGPTV-Kan (16) and transferred into the *Agrobacterium tumefaciens* strains GV3101 (17) or LBA4404 (18). Transformation of tomato leaf explants was done as described (19).

DNA Sequencing and Analysis. DNA sequencing was done by using the PRISM Ready Reaction Terminator Cycle Sequencing system (Applied Biosystems). Reactions were run on an Applied Biosystems 373A or 377XL DNA sequencer. Computer analysis was performed by using the following software: WISCONSIN package, Version 9.1, Genetics Computer Group, Madison, WI.

RESULTS

Establishment of a Cosmid Contig from the *ls* Region. Previous work had mapped the *Ls* locus to an interval with a maximum size of 375 kb at the top of chromosome 7 defined by the restriction fragment length polymorphism markers CD61 and CD65 (8). Subsequently, CD61-specific primers were used to screen a pooled tomato YAC library (14). Among five clones isolated, YAC CD61–5 was shown by Southern analysis to hybridize to both CD61 and CD65, and therefore its 320-kb insert should encompass the whole CD61-CD65 interval including the *Ls* locus.

DNA of YAC CD61–5 was isolated by pulsed field gel electrophoresis and used as a probe to screen a tomato shoot tip cDNA library (12). Among the isolated cDNA clones, representing at least 29 transcripts, only one, y25, showed cosegregation with the *Ls* locus and was therefore used as an anchor to establish a cosmid contig of the *Ls* region on chromosome 7. Screening of a genomic cosmid library with y25 resulted in an initial set of four overlapping cosmid clones. To orient this set of overlapping cosmid clones relative to the genetic map, end probes of the contig were mapped in a population of recombinants harboring recombination breakpoints around the Ls locus (8). Whereas probe C-for detected three recombinants in the interval between *Ls* and CD65, the end probe A-rev of cosmid A cosegregated with the *Ls* locus (Fig. 1*A*), indicating that we had to extend the contig in the direction of CD61. After two consecutive rounds of isolating overlapping cosmid clones, a contig covering ≈60 kb of genomic DNA was established (Fig. 1*A*).

Functional Complementation of the ls^1-Mutant. To test for complementation, cosmids (Fig. 1*A*) were introduced into leaf discs of the *lateral suppressor* mutant (ls^1/ls^1) by using the *Agrobacterium* strain LBA4404. Transgenic plants were screened for development of side-shoots and petals. A total of

50 transgenic plants harboring the cosmids A, B, C, D, E, and F or the vector plasmid pCLD04541 produced neither side-shoots nor petals (Table 1). However, eight of 16 independent transgenic plants transformed with either cosmid G or cosmid L did complement the mutant phenotype. Southern blot analysis revealed that only those plants showing complementation contained a complete T-DNA copy. Three transgenic plants harboring an intact copy of cosmid G developed side-shoots in almost every leaf axil and produced a whorl of petals on all flowers (Fig. 2). In contrast, we found that the five transgenic lines harboring a complete copy of cosmid L developed side-shoots in only a fraction of their leaf axils (69% in one transgenic line) and also showed an incomplete restoration of the flower phenotype. Because this finding may indicate that the *Ls* gene of cosmid L contains a mutation or that a regulatory element is missing, subsequent experiments were done by using cosmid G.

To define the position of the *Ls* gene more precisely, we tested subfragments of cosmid G for complementation of the ls^1 mutant. Whereas introduction of the 10.3-kb *Cla*I fragment (Fig. 1*B*) did not complement the ls^1 mutant, the subfragments GSET 4 (8.4 kb) and GSET 6 (5.6 kb) restored the wild-type phenotype (Table 1). This result demonstrated that the *Ls* gene is located within the 5.6-kb *Sna*BI–*Xho*I fragment of cosmid G.

Inheritance of the complementation phenotype was analyzed in the transgenic line 9620 harboring a single copy of cosmid G. Among 28 plants of the self-pollinated progeny of 9620, we found 20 plants showing complementation and eight plants with the *ls* phenotype. Resistance to kanamycin was observed only in those plants showing complementation. This result is consistent with the assumption that a single-copy T-DNA insertion, segregating in a Mendelian fashion, rescues the *ls* phenotype.

Identification and Characterization of the *Ls* gene. Subfragments covering almost the complete cosmid G were used as probes to screen a shoot tip cDNA library prepared from RNA of vegetative and floral shoot tips. Among 10^6 clones tested, we identified two cDNA clones of which only one, ET1, was found to be derived from the 5.6-kb *Sna*BI–*Xho*I fragment showing complementation of the ls^1 mutant. DNA sequence analysis revealed that ET1 contains an ORF starting with the first nucleotide of the cDNA and ending with a stop codon at position 1415 (GenBank accession no. AF098674), followed by an untranslated 3′ region of 271 bp, and a poly(A) tail.

To determine the 5′ end of the transcript, three independent products obtained in 5′ RACE experiments were sequenced. All three products started at the same base pair (position 1), suggesting that this position corresponds to the 5′ end of the transcript. The ATG initiating the ORF (position 131) is preceded by several stop codons in all three frames, strongly suggesting that this ATG corresponds to the translation start site. From these experiments, we conclude that the *Ls* transcript has a length of ≈1,7 kb and contains an ORF with a coding capacity for 428 aa. Comparison of the cDNA and the corresponding sequence of the genomic DNA revealed no sequence deviation between the cDNA and its genomic counterpart, which demonstrates that the *Ls* gene does not contain an intron.

Table 1. Complementation experiments

Construct	No. of transgenic plants	No. of plants showing complementation
pCLD04541	8	0
Cosmid A	5	0
Cosmid B	15	0
Cosmid C	5	0
Cosmid D	7	0
Cosmid E	2	0
Cosmid F	8	0
Cosmid G	5	3
Cosmid L	11	5
GSET4	2	2
GSET6	13	13
*Cla*I	5	0

To prove that the ORF identified corresponds to the *Ls* gene, we searched for sequence alterations in the mutant *ls* alleles. For this purpose, PCR products derived from genomic DNA of the ls^1 and ls^2 mutant were sequenced. A deletion of ≈1.5 kb was detected in ls^1, removing the first 185 aa of the predicted protein and 995 bp of the leader and the presumptive promoter region. In the ls^2 allele, the nucleotide sequence CAACAGCG (position 203–210) is replaced by TA-AAAACGGAA. The C to T transition at position 203, which changes a Q to a stop codon, is predicted to cause a premature termination of translation after 24 aa. The results of the complementation experiments together with the sequence analysis of the wild-type and the two mutant *ls* alleles demonstrate that we have isolated the *Ls* gene.

Ls Is a New Member of the VHIID Family. Comparison of the Ls protein sequence to the databases resulted in a list of proteins with considerable sequence similarity. Besides several sequences of unknown function identified in sequencing projects, this list includes three genes identified recently in *Arabidopsis thaliana*, which belong to the VHIID family of regulatory proteins: Scarecrow (SCR; ref. 20), Gibberellin insensitive (GAI; ref. 21), and repressor of gal-3 (RGA; ref. 22). With the exception of the N terminus (amino acids 1–47) similarity between Ls and the other members of this family extends over the whole length of the ORF (≈35% sequence identity by using the computer program FASTA). As we have recently isolated a clone from *A. thaliana* with considerably higher sequence similarity to *Ls* than either *SCR, GAI,* or *RGA* (unpublished results), we can exclude the possibility that *Ls* represents the tomato homolog of one of these genes.

The VHIID motif of unknown function, after which this family of proteins was named, is included in a modified version (IHIVD) in a region (amino acids 152–191 in Ls) showing the highest conservation between the different members of the family (Fig. 3). Only two additional sequence motives described for one or more of the three related proteins are conserved in Ls: the second leucine heptad repeat found in all four related genes and the LXXLL-motif (^{267}LHRLL^{271}) found in GAI and RGA that was recently shown to mediate interaction of transcriptional coactivators with nuclear receptors (23). All other sequence motives described for one or more of the related proteins, like the nuclear localization signal, the first leucine heptad repeat, or the bZIP-like domain, are not found in Ls, which makes a functional conservation unlikely.

The N terminus of the Ls protein is considerably shorter than the N termini of the three related proteins (47 aa in Ls, 288 aa in SCR, 166 aa in GAI, and 219 aa in RGA), and it does not show obvious sequence conservation, but it shares with the other sequences clusters of serine and threonine residues (amino acids 7–46). A second region with clustered serine and threonine residues is found in Ls between amino acids 106 and 128.

RT-PCR Detection of *Ls* mRNA. The identification of only one hybridizing cDNA clone from a shoot tip cDNA library among ≈10^6 plaques tested suggested that the steady–state levels of the *Ls* transcript are very low. This initial observation was corroborated by the finding that the *Ls* mRNA was not detectable in Northern blot hybridization experiments. Therefore, RT-PCR analysis was performed with total RNA from different plant organs. The *Ls* transcript was detected in shoot tips, flowers, roots, and young leaves but not in internodes (Fig. 4*A*). Because of the inherent characteristics of the RT-PCR technique, the observed

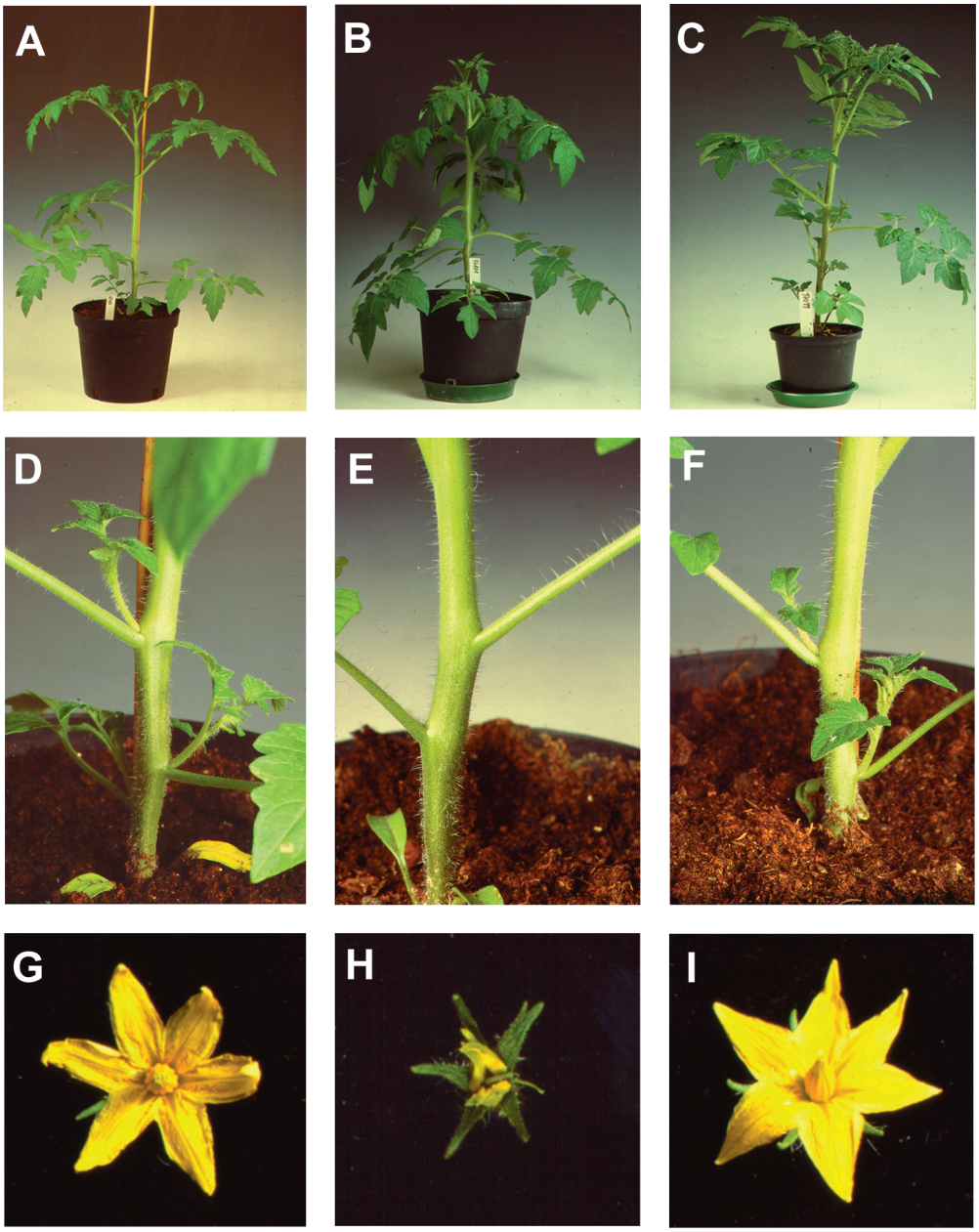

FIG. 2. Functional complementation of ls^1. Comparison of phenotypes of Antimold B (*A*, *D*, and *G*), Antimold B-ls^1 (*B*, *E*, and *H*), and Antimold B-ls^1 transformed with cosmid G (*C*, *F*, and *I*). The pictures show the growth habit (*A-C*), a close up of leaf axils (*D-F*), and a close-up of a flower (*G-I*).

quantitative differences in mRNA levels have to be treated with caution.

To test for the presence of a transcript from the two mutant alleles, total RNA extracted from young leaves of plants homozygous for either the wild-type *Ls* allele, ls^1, or ls^2, respectively, was analyzed by RT-PCR. In ls^1/ls^1 plants, no mRNA was detected whereas ls^2/ls^2 and wild-type plants contained transcripts of equal size (Fig. 4*B*). This result is in agreement with the finding that the ls^1 allele contains a deletion removing part of the ORF as well as the presumptive promoter region and demonstrates that ls^1 is clearly a null allele.

DISCUSSION

We have isolated the *Ls* locus by positional cloning and demonstrated the identity of the *Ls* gene by complementation and sequence analysis of the two existing mutant alleles. The identical phenotypic defects of the mutant alleles are in both cases because of a complete loss of function of the Ls protein caused by a deletion in ls^1 and the introduction of a stop codon after only 24 aa in ls^2.

The protein encoded by the *Ls* gene shares significant sequence similarity with members of the emerging family of plant VHIID proteins. In addition to the characteristic VHIID domain of unknown function, the previously described members of this family, SCR (20), GAI (21), and RGA (22), show a number of features pointing to a potential role as transcriptional regulators. In the well conserved C-terminal part only a leucine heptad repeat (amino acids 206–226) and an LXXLL (aa 267–271) motif are conserved between Ls and other family members. The LXXLL motif has been shown to be involved in binding of steroid receptor coactivators to the respective steroid receptors (23). However, the significance of this motif for plants is questionable because it occurs frequently and the presence of nuclear receptor-like proteins in plants has yet to

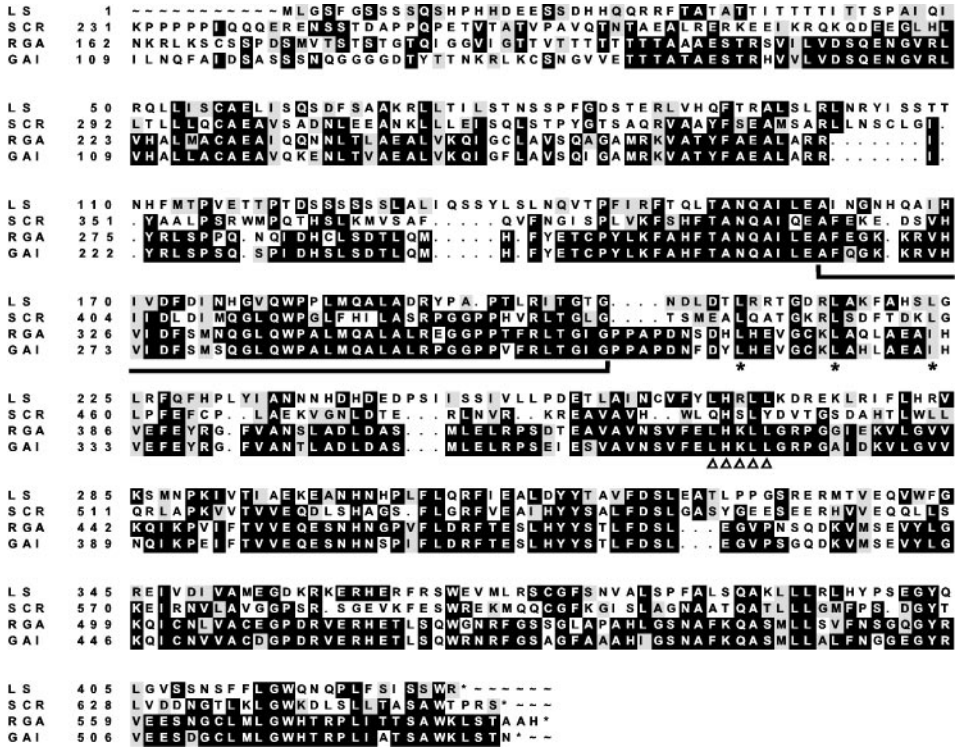

FIG. 3. Sequence analysis of the *Ls* gene. Alignment of the amino acid sequences of the Ls protein with the *Arabidopsis thaliana* proteins SCR (19), RGA (21), and GAI (20). Identical residues are displayed in reverse type and similar residues are in gray boxes. The VHIID domain is underlined, the leucine residues defining the conserved leucine heptad repeat are identified by asterisks, and the LXXLL motif by triangles.

be shown. Although the N-terminal domains of members of this family are of varying length and are not similar in sequence, the presence of short homopolymeric stretches of serine and threonine residues is a feature that the Ls protein shares with RGA and SCR and to a lesser extent with GAI. Such homopolymeric stretches have been found in the activation domains of transcription factors, and it has been demonstrated that serine- or threonine-rich stretches can serve as targets for the modification with *N*-acetylglucosamine residues leading to changes in activity of the respective proteins (24). Such modifications may be introduced by proteins like the SPINDLY protein of *A. thaliana*, which is involved in the regulation of GA signal transduction and shares sequence homology with *N*-acetylglucosamine transferases from animals (25). As the predicted Ls protein lacks a putative NLS signal, we do not have strong indications for a role as a transcriptional regulator. The presence of a conserved leucine heptad repeat however makes it conceivable that it interacts with related proteins, which themselves act as transcriptional regulators.

The fact that the protein encoded by the *Ls* gene is related to two proteins involved in negative regulation of GA signal transduction (GAI, RGA) lends support to a model that postulates a role for the Ls protein in a mechanism of localized regulation of GA responsiveness. This view is supported by the finding that the *ls* mutant is characterized by a severe imbalance of the major plant hormones (7). Among the hormones that show altered levels, GA is of particular interest as some aspects of the *ls* phenotype, like reduced seed germination and petal development, are known to be influenced by GA (26, 27). To maintain their undifferentiated state, meristematic cells must be protected from hormonal signals inducing differentiation in surrounding cells. One way to achieve this is through a localized negative regulation of the GA signal transduction pathway. The low abundance of the *Ls* mRNA has so far not allowed us to support this model by analyzing the expression on the *in situ* level, but RT-PCR analysis shows *Ls* expression

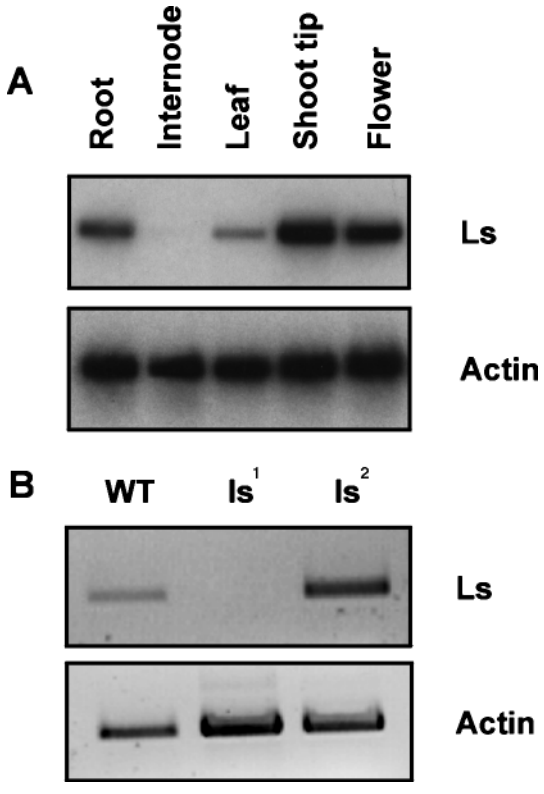

FIG. 4. RT-PCR detection of *Ls* mRNA in different plant organs. (*A*) Total RNA from different plant organs was analyzed by RT-PCR, and the PCR products were hybridized to the *Ls* cDNA as probe. Amplification of actin cDNA was used to ensure that equal amounts of cDNA were added to each PCR reaction. (*B*) Total RNA was extracted from leaves of plants homozygous for Antimold B-*Ls* (Wt), Antimold B-*ls*^{1}, and Primabel-*ls*^{2} and then analyzed by RT-PCR.

in tissues that include the primordia affected in *ls* mutant plants. The dramatic increase in GA levels found in different organs of the *ls* mutant (7) could be ascribed to a perturbation of feedback inhibition of GA synthesis as it is assumed in the cases of GA-insensitive mutants, which contain elevated levels of GA (28, 29). The imbalances in the levels of the other major plant hormones may be either the result of an interrelation of the metabolisms of the different plant hormones or may indicate that the Ls protein is involved in different signal transduction pathways. The isolation of the *Ls* gene allows us to test our model and address the question of the causal relationship between morphological defects and hormonal imbalances on the molecular level.

Despite carrying null alleles, *ls* mutants are still able to form axillary meristems in the axils of the two leaf primordia preceding the inflorescence. This observation indicates that the Ls protein is not absolutely required for axillary meristem formation. It seems possible that, with the transition of the vegetative shoot apical meristem into an inflorescence meristem, the strength of the differentiation signal is reduced so that a protection of the cells forming the axillary meristem is no longer needed. Alternatively, it could be assumed that the mechanisms underlying the formation of the sympodial sideshoots are different and that the *Ls* gene is not involved in this process.

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