Planta (1995) 195:541–547

Planta


# Isolation and characterization of the tomato homeobox gene *THOM1*

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Received: 13 April 1994 / Accepted: 28 June 1994

**Abstract.** We have screened a cDNA library prepared from tomato (*Lycopersicon esculentum* Mill. cv. VFNT Cherry) shoot tips for homeobox-containing clones. The isolated cDNA clone THOM1 (tomato homeobox gene 1) contains a homeobox, a leucine zipper motif and and an acidic region. These features support the hypothesis that the *THOM1* gene product acts as a transcriptional activator. *THOM1* is highly expressed in the vegetative shoot apical meristem, the floral meristem and axillary meristems. Young derivatives of these meristems show similar levels of THOM1 transcripts which decrease with increasing age of the respective tissue. The *THOM1* gene is located in the centromere region of chromosome 1 and belongs to a small gene family of the tomato genome.

**Key words:** HD-Zip protein – Homeobox – *Lycopersicon* – Shoot apical meristem

## Introduction

The growth habit of higher plants is determined to a large extent by activities of the apical meristems. The shoot apical meristem (SAM) continuously produces new cells which build the aerial part of the plant. Frequency and orientation of cell divisions in the SAM are highly regulated in space and time, resulting in a species-specific pattern of organ initiation (Lyndon 1990). Whereas the descendant cells differentiate to form the different tissues and organs of the shoot system, the shoot apical meristem is preserved as a functional unit during the whole life cycle of the plant (Steeves and Sussex 1989). In addition, the SAM controls growth and differentiation of nearby and remote parts of the shoot by hormonal signals (e.g. apical dominance, Shabde and Murashige 1977). The activities of the SAM imply the existence of an underlying developmental program governed by genetic control elements.

Different approaches have been used to identify genes involved in the genetic control of activities of the SAM. Mutants affected in different aspects of meristem organization and function have been identified in several plant species (e.g. Caruso 1968; Mayer et al. 1991; Medford et al. 1992; Barton and Poethig 1993). The analysis of such mutants has allowed stages of meristem development to be defined (Sussex 1989; Medford et al. 1992). It is, however, difficult to isolate the corresponding genes, because their gene products are not known. Several groups have used the technique of differential screening to isolate genes that are preferentially or exclusively expressed in the SAM. Characterization of the isolated cDNA sequences revealed that most of the corresponding genes encode 'housekeeping' functions (e.g. histones) typical of tissues exhibiting high rates of cell division (Medford et al. 1991; Köhler et al. 1992; Pri-Hadash et al. 1992; Shahar et al. 1992). So far, this approach has not been successful in isolating regulatory genes for vegetative plant development.

Genes known to play an important role in regulation of developmental processes in several organisms are the homeobox genes, first discovered in *Drosophila melanogaster* (Gehring et al. 1987). This class of genes is characterized by a conserved sequence motif, the homeobox, encoding a sequence specific DNA-binding domain, the homeodomain (HD; Desplan et al. 1988; Hoey and Levine 1988; Müller et al. 1988). A typical homeodomain consists of three or four α-helices, the second and third of which are connected by a turn (Quian et al. 1989; Kissinger et al. 1990; Otting et al. 1990). After their discovery in *Drosophila*, homeobox genes have been identified in several organisms, including various animal species (Scott et al. 1989; Schummer et al. 1992), yeast (Shepard et al. 1984), fungi (Schulz et al. 1990) and higher plants (Ruberti et al. 1991; Vollbrecht et al. 1991; Bell-

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Abbreviations: HD-Zip protein = protein containing a homeodomain and a leucine zipper; RFLP = restriction fragment length polymorphism; SAM = shoot apical meristem

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mann and Werr 1992; Schena and Davis 1992). Despite the differences in the developmental programs of these species, many homeobox genes proved to be important regulators of developmental processes (Harvey and Melton 1988; Le Mouellic et al. 1992; Smith et al. 1992). For example, the *Knotted-1* gene from *Zea mays* is involved in the regulation of cell determination (Smith et al. 1992; Sinha et al. 1993), while *HAT4*, a homeobox gene from *Arabidopsis thaliana*, seems to influence the developmental pace of the plant (Schena et al. 1993).

Homeobox genes expressed in the shoot apical meristem are candidates for a regulatory role in the SAM. To isolate such genes, we have screened a tomato shoot tip cDNA library for cDNAs containing the homeobox motif. This allowed the isolation and characterization of *THOM1*, the first tomato gene of the HD-Zip protein (proteins containing a homeodomain and a leucine zipper) family (Ruberti et al. 1991).

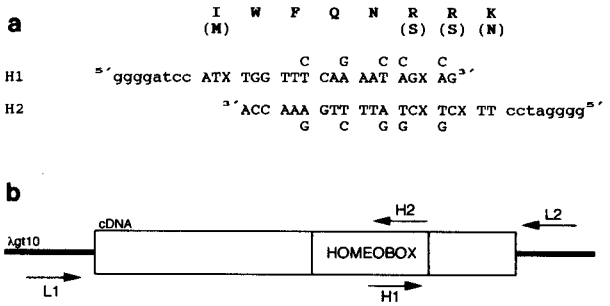


**Fig. 1a,b.** Screening for cDNA clones containing the homeobox motif. **a** Nucleotide sequence of the degenerate oligonucleotides H1 and H2. Both oligonucleotides are derived from the amino-acid sequence of the conserved region of the recognition helix (*top line*), H2 is complementary to the coding, H1 to the noncoding strand. Both primers are equipped with a *Bam*HI cleavage site. **b** Schematic drawing of the control PCR reaction

## Materials and methods

*Plant material.* Tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker and VFNT Cherry) seed material was obtained from the Tomato Genetics Stock Center, Davis, Calif., USA. Plants were grown under standard glasshouse conditions with additional artificial light (16 h photoperiod) during the winter period.

*Library screening and cDNA isolation.* A tomato (cv. VFNT Cherry) shoot tip cDNA library containing $3 \cdot 10^6$ recombinants in the phage vector λgt10 (Simon 1990) was prepared from RNA of tomato shoot apices. Besides the apical meristem the tissue collected for RNA extraction included two to three leaf primordia. The library was screened with the degenerate oligonucleotide H1 (Fig. 1a) as described by Bürglin et al. (1989). The polymerase chain reactions (PCRs) were performed using the following PCR regime: 40 s/95°C, 2 min/55.5°C, 2 min/72°C (35 ×), and 10 min elongation at 72°C.

*Manipulation of nucleic acids.* Restriction analysis, gel electrophoresis, DNA cloning and other standard techniques were used as described by Sambrook et al. 1989. Sequencing of both DNA strands was performed using the fmol sequencing kit (Promega) according to the manufacturer's instructions.

*Preparation of DNA and Southern blot analysis.* The DNA was prepared as described by Brandstädter et al. (1994). For Southern blot analysis, approx. 5 µg of genomic DNA was subjected to electrophoresis through 0.8% agarose. The DNA was blotted onto Hybond $N^+$ membranes (Amersham Buchler, Braunschweig, Germany) according to manufacturer's instructions. Hybridization was performed either under standard conditions (Sambrook et al. 1989) or under low-stringency conditions (at 30°C in standard hybridization solution with 50% formamide; washing up to 1 × SSPE (150 mM NaCl, 10 mM $NaH_2PO_4$, 1 mM EDTA, pH 7.4), 0.1% SDS at 60°C).

*Preparation of RNA and Northern blot analysis.* Total RNA was prepared following the method of Chomcyznski et al. (1987). For Northern blot analysis approx. 20 µg RNA were loaded onto denaturing gels. After electrophoresis, the RNA was transfered onto Hybond-$N^+$ membranes and hybridized in 50% formamide at 42°C as described by Sambrook et al. (1989).

*Restriction fragment length polymorphism (RFLP) mapping.* The THOM1 cDNA was used to probe a Southern blot of *L. esculentum* and *L. pennellii* DNA separately digested with *Bam*HI, *Dra*I, *Hind*III, *Xba*I, *Eco*RI, and *Eco*RV to identify an RFLP. Subsequently, the probe was hybridized to *Eco*RV-digested DNA of 33 F2 plants derived from an *L. esculentum* (cv. VF36) × L. pennellii F1-hybrid. Segregation data were analyzed using the computer program MapMaker (Lander et al. 1987) and a framework dataset of 335 RFLP markers distributed over the 12 tomato chromosomes (Tanksley et al. 1992).

*In-situ RNA hybridizations* were performed as described by Brandstädter et al. (1994). The antisense strands of the *Apo*I restriction fragment (bp 105–1107, Fig. 2) containing the coding region of *THOM1* and the 486 bp 5′-fragment (bp 1–486, Fig. 2) were used as RNA probes. The sense strand of the 486-bp 5′-fragment was used as a negative control. Slides were developed after three weeks exposure of the photoemulsion.

*Computer analysis* was done using the UWGCG software (Devereux et al. 1984) on a vax workstation.

## Results

*Isolation of cDNA clones.* A shoot tip cDNA library from *Lycopersicon esculentum* (cv. VFNT Cherry) was screened for clones containing the homeobox motif following the procedure of Bürglin et al. (1989). The degenerate oligonucleotide H1 (Fig. 1) deduced from the amino-acid sequence of the highly conserved recognition helix was used as a probe. Hybridizing λ-clones were tested again for the presence of the homeobox by PCR analysis using the oligonucleotides H1 or H2 (Fig. 1) in combination with the primers L1 or L2, which border the cloning site in the phage vector λgt10. One of the cDNA clones yielded amplification products with both pairs of primers. The sum of the sizes of these two DNA fragments was equal to the size of the original cDNA insert. The respective cDNA, designated THOM1 (tomato homeobox gene 1), was isolated as an *Eco*RI restriction fragment and cloned into the plasmid vector pUC19.

*Sequence analysis.* The cDNA clone THOM1 has a length of 1241 bp including a polyA-tail of 29 bp. It contains one long open reading frame, starting with the first ATG at position 151 and terminating with a stop codon at position 1009. The presumptive protein has a length of

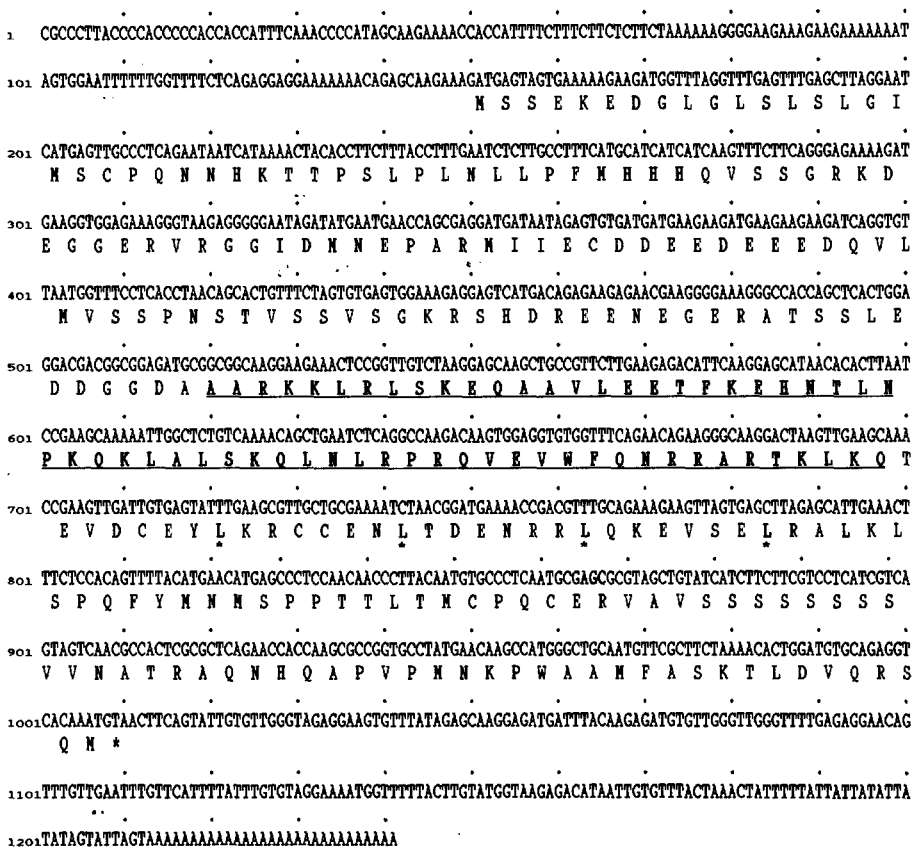

**Fig. 2.** Nucleotide sequence and deduced amino-acid sequence of the *THOM1* cDNA. *Upper line*, nucleotide sequence; *bottom line*, deduced amino-acid sequence, indicated in the one-letter code. The homeobox sequence is *underlined*; leucine residues of the proposed leucine zipper are marked by *asterisks*

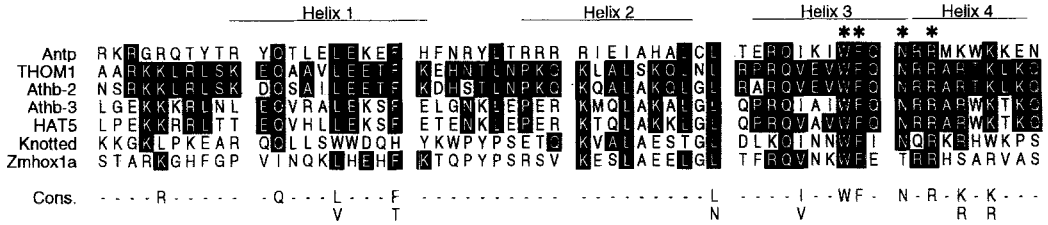


**Fig. 3.** Sequence comparison of the *THOM1* homeodomain with other homeodomains. HD-ZIP proteins from *Arabidopsis thaliana*: Athb2, Athb3, and HAT5; homeobox genes from *Zea mays*: *Knotted* and *Zmhox1a*; *Antp*, homeodomain of the *antennapedia* gene from *Drosophila melanogaster*; *Cons*, homeodomain consensus sequence. The four invariant amino acids of the homeodomains are marked by *asterisks*. Amino acids that are identical to THOM1 are *shaded*

286 amino acids (Fig. 2) and revealed similarities to several known sequence motifs. Between amino acids 124 and 183 the polypeptide contains a homeodomain, as shown by alignment with the antennapedia homeodomain (Gehring et al. 1992; Fig. 3). The THOM1 homeodomain contains the four invariant amino acids and seven of the eight highly conserved amino acids that are characteristic of homeodomains (Scott et al. 1989). The remaining highly conserved amino acid is replaced by an isomorphic substitute. The region downstream of the homeodomain is characterized by a series of four leucine residues each separated by six amino acids (aa 193–215). This configuration is known from several transcription factors and was termed the 'leucine zipper motif' (Landschulz et al. 1988).

The combination of a homeodomain and a leucine zipper motif is the characteristic feature of a class of proteins discovered in *Arabidopsis thaliana* which were termed HD-Zip proteins (Ruberti et al. 1991). Sequence comparisons revealed that *THOM1* has the highest similarity to Athb-2 (Carabelli et al. 1993); HAT4 (Schena and Davis 1992), another member of the HD-Zip family, differs from Athb2 only by a single amino-acid substitution. The similarity to Athb-2 is very high in the central part of the protein [amino acids (aa) 123–242] containing the homeobox-leucine zipper region (83% identity/91% similarity), whereas it is moderate in the N-terminal region (aa 1–122, 35% identity/52% similarity) and low in the part close to the C-terminus (aa 243–286, 23% identity/38% similarity) of the protein.

N-terminal to the homeodomain, the putative THOM1 protein contains an acidic region (aa 50–121) with a conspicuous stretch of nine consecutive acidic amino acids (aa 74–82). It has been shown that such

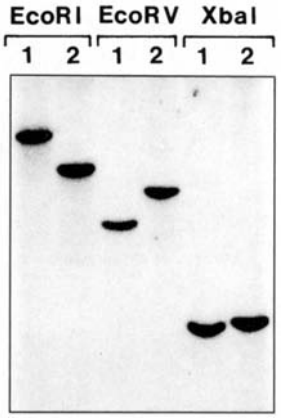


**Fig. 4.** Southern blot analysis of THOM1-related sequences in tomato genomic DNA. The DNA was prepared from *Lycopersicon esculentum* cv. Moneymaker (*lanes 1*) and *L. pennellii* (*lanes 2*), and hybridized under standard hybridization conditions with the radioactively labeled THOM1 cDNA. Restriction enzymes are indicated above adjacent lanes

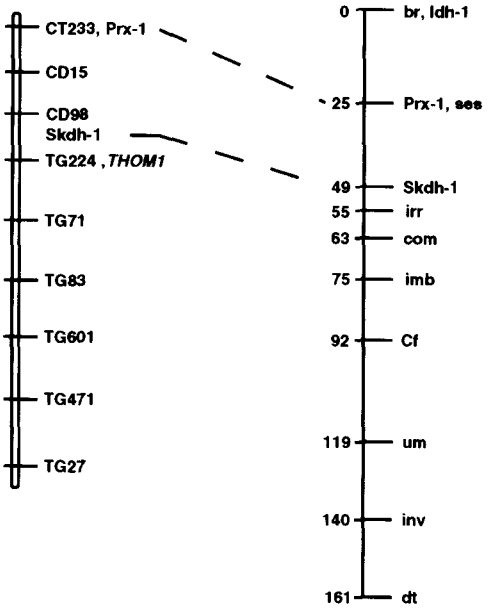


**Fig. 5.** Restriction fragment length polymorphism map position (*left*) of chromosome 1 and comparison with classical map (*right*). The map position of THOM1 is indicated on a partial RFLP map of chromosome 1 of tomato. Framework RFLP markers and morphological markers (Tanksley et al. 1992) are indicated beside tick marks

acidic segments can interact with RNA polymerase II and constitute the domain that activates transcription (Mitchell and Tjian 1989).

*Southern analysis and genomic organization.* To determine the copy number of *THOM1*-related sequences in the tomato genome, Southern blot hybridization experiments were performed using the THOM1 cDNA as a probe. At high stringency *THOM1* behaved as a single copy gene. As shown in the autoradiograph (Fig. 4), only one hybridizing DNA fragment was detected following digestion of tomato genomic DNA with several restriction enzymes. Under reduced stringency conditions, one prominent band and four additional weaker bands were observed (data not shown). These experiments suggest that the *THOM1* gene is a member of a small gene family.

To localize the *THOM1* gene within the tomato genome, we have analyzed DNA of 33 plants of a segregating F2 population derived from a cross between *L. esculentum* (cv. VF36) and *L. pennellii* for RFLPs. An *Eco*RV polymorphism (Fig. 4) allowed us to establish that the *THOM1* gene cosegregates with the RFLP marker TG224 (Tanksley et al. 1992), which maps to the centromere region of chromosome 1 (Fig. 5). Therefore, *THOM1* is located in the same chromosome 1 segment as the genes *irregularis* (*irr*), *complicata* (*com*), and *imbecilla* (*imb*; Tanksley et al. 1992), which, upon mutation, alter the morphology of the leaf (*com*, *irr*) or the plant habit (*imb*). In a preliminary Northern experiment with RNA from young leaves (0.5–1 cm) of these mutants the THOM1 cDNA hybridized to a RNA indistinguishable in size and amount from the wild-type transcript (data not shown). However, this result does not exclude the THOM1 cDNA being derived from one of these genes.

*Analysis of THOM1 mRNA distribution.* The steady-state level of THOM1 mRNA was analyzed in Northern blot experiments probing total RNA isolated from various plant tissues with the THOM1 cDNA. A transcript of

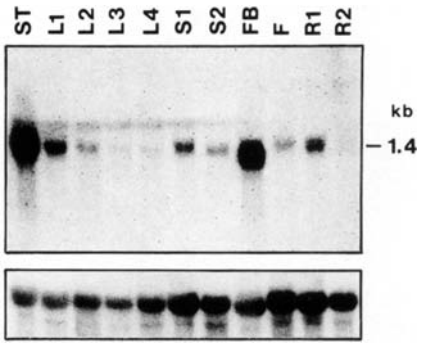


**Fig. 6.** Northern blot analysis of RNA from different plant tissues. *Upper part*: Twenty µg of total RNA, as estimated by $OD_{260nm}$ measurements, from different tissues at different developmental stages was hybridized with the THOM1 cDNA. *ST*, shoot tips from 13-d-old plants; *L1*, leaves smaller than 2 cm; *L2*, leaves between 3 and 5 cm; *L3*, leaves between 5 and 10 cm; *L4*, adult leaves (>20 cm); *S1*, stem, 1 mm diameter; *S2*, stem, 3 mm diameter; *FB*, flower buds; *F*, flower; *R1*, roots from 7 d old plants; *R2*, roots from adult plants. *Lower part*: The same blot was rehybridized with an 18S rRNA probe as a control for the amount of RNA

1.4 kb was detected in highest amounts in RNA preparations from the shoot tip and young flower buds (Fig. 6). Reduced levels of the same transcript were found in developing leaves, stems, flowers, and roots. With increasing age of these tissues, the signals decreased to a low level that was observed in every adult tissue tested.

The expression pattern of *THOM1* was further analyzed by RNA in situ-hybridization experiments. The THOM1 mRNA was found to be homogenously distributed throughout the shoot apical meristem (Fig. 7a).

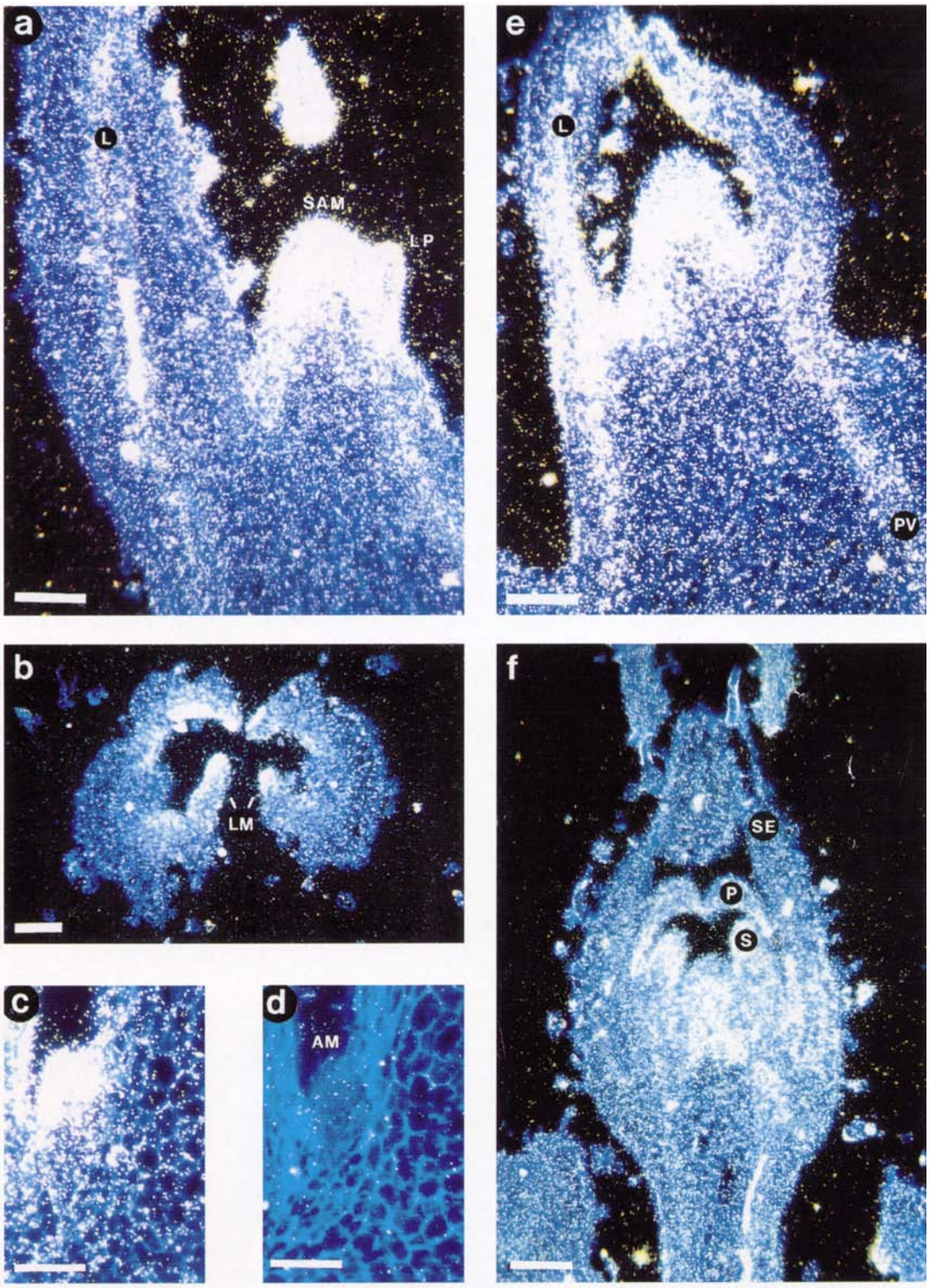


**Fig. 7a–f.** In-situ localization of THOM1 transcripts in tomato shoots. Longitudinal sections of vegetative shoot tips (**a**), axillary buds (**c**), shoot tips at the transition to flowering (**e**), young flowers (**f**) and transverse sections of young leaves (**b**) were hybridized with the antisense strand of a 5′-fragment (bp 1–486) of the THOM1 cDNA clone or with a mixture of the 5′-fragment and the ApoI-fragment (bp 106–1107). **d** Shows a longitudinal section of an axillary bud hybridized to the sense strand of the THOM1 5′-fragment (negative control). Autoradiographic signals were studied in dark-field illumination and epifluorescence was used to visualize the underlying tissue. ×110 (**a**, **c**, **d**, **e**), ×100 (**f**), ×75 (**b**); bars = 100 μm

However, the signal was not restricted to the SAM: it extends into the underlying rib meristem and fades in the differentiating stem tissue and the developing vascular bundles. We also detected THOM1 transcripts in leaf primordia (Fig. 7a) and young leaves, which showed prominent staining primarily at the adaxial side and the rims of the lamina (Fig. 7b). In older leaves the signal was restricted to developing leaflets (data not shown) and it decreased until maturation (compare with Northern analysis, Fig. 6). Strong hybridization signals were also observed in axillary buds (Fig. 7c). After the transition to reproductive growth, transcription of THOM1 was maintained in the floral meristem (Fig. 7e) and in all organs of the young flower (Fig. 7f). As shown also by Northern experiments, the THOM1 signal fades during flower maturation to very weak intensity (Fig. 6). Mature tissues revealed only very weak hybridization signals or no signal above background.

## Discussion

To study regulatory genes involved in the control of activities of the shoot apical meristem, we have screened a shoot tip cDNA library for clones containing the homeobox motif. Here we report the isolation and characterization of *THOM1*, a gene that is characterized by the presence of a homeobox and a closely linked leucine zipper motif. *THOM1* shows similarity to *Athb-2* (Carabelli et al. 1993) and *HAT4* (Schena and Davis 1992), two members of the HD-Zip protein family from *A. thaliana*, which differ only by a single amino-acid substitution. Because of the low degree of homology in the N-terminal and C-terminal regions it seems unlikely that *THOM1* represents the tomato homolog of the *Athb-2* or the *HAT4* gene from *A. thaliana*. The observed sequence similarities indicate that the *THOM1* gene is a new member of the HD-Zip protein family.

Most of the homeobox genes isolated so far encode proteins that bind to DNA as monomers. However, recently it has been shown that the HD-Zip proteins Athb-1 and Athb-2 recognize dyad-symmetric DNA sequences as homodimers formed via dimerization of the leucine zippers (Sessa et al. 1993). Therefore, the presence of a homeobox and a leucine zipper motif in the THOM1 cDNA suggests that *THOM1* encodes a DNA-binding protein that may also act as a dimer. Because several members of the HD-Zip protein family are present in the tomato genome (data not shown), it is tempting to speculate that, beside homodimers, heterodimers may also be formed, which could greatly increase the flexibility of regulatory interactions.

The distribution of THOM1 transcripts in the shoot apex resembles the distribution of total RNA as observed after acridine-orange staining. This correlation raises the question, whether the stronger signal in this tissue is due to a general increase in RNA concentration or reflects an increased level of THOM1 transcripts relative to other transcripts. The Northern analysis demonstrates that indeed the relative concentration of THOM1 mRNA is considerably higher in undifferentiated than in mature tissues (Fig. 6). The observed distribution of THOM1 mRNA resembles to a certain extent the distribution of Knotted-1 (Kn-1) protein in maize (Smith et al. 1992). However, different from Kn-1, THOM1 mRNA is clearly detectable in lateral organs derived from the SAM. During development of these organs *THOM1* transcription is downregulated to low levels with the exception of areas exhibiting high rates of cell division. The observed distribution of THOM1 mRNA suggests that this gene is probably not a regulator directing development towards a particular organ type but that its expression is correlated with the capability to undergo cell division.

Despite the large number of homeobox genes isolated from different animal species, the HD-Zip motif has been found so far only in plant genes. Carabelli et al. (1993) have demonstrated that *Athb-2* and *Athb-4*, two members of the HD-Zip protein family, are inducible by far-red-light. In addition, seeds from transgenic plants overproducing the *HAT4* gene, another member of the HD-Zip family, exhibit a light-reversible germination defect (Schena et al. 1993). Taken together, these findings indicate that this group of regulatory proteins may be involved in the control of plant specific processes, which might be triggered by light. Antisense inhibition and ectopic expression of *THOM1* in transgenic plants will be useful tools to study the function of *THOM1* during plant development.

The authors thank Dr. Steven Tanksley (Cornell University, Ithaca, N.Y., USA) for providing the segregating F2 population and a framework data set. We are also grateful to Drs. Peter Starlinger, Wolfgang Werr (Institut für Genetik, Universität zu Köln, Germany) and Francesco Salamini (MPI für Züchtungsforschung, Köln, Germany) for critical reading of the manuscript, and members of the laboratory for useful discussions. This work was supported by the Bundesministerium für Forschung und Technologie.

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