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Regulation of meristem organization and cell division by *TSO1*, an *Arabidopsis* gene with cysteine-rich repeats

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SUMMARY

In higher plants, meristem organization and cell division regulation are two fundamentally important and intimately related biological processes. Identifying and isolating regulatory genes in these processes is essential for understanding higher plant growth and development. We describe the molecular isolation and analyses of an *Arabidopsis* gene, *TSO1*, which regulates both of these processes. We previously showed that *tso1* mutants displayed defects in cell division of floral meristem cells including partially formed cell walls, increased DNA content, and multinucleated cells (Liu, Z., Running, M. P. and Meyerowitz, E. M. (1997). *Development* 124, 665-672). Here, we characterize a second defect of *tso1* in inflorescence meristem development and show that the enlarged inflorescence in *tso1* mutants results from repeated division of one inflorescence meristem into two or more inflorescence meristems. Using a map-based approach, we isolated the *TSO1* gene and found that *TSO1* encodes a protein with cysteine-rich repeats bearing similarity to *Drosophila Enhancer of zeste* and its plant homologs. In situ *TSO1* mRNA expression pattern and the nuclear localization of TSO1-GFP are consistent with a regulatory role of *TSO1* in floral meristem cell division and in inflorescence meristem organization.

Key words: *TSO1*, Meristem organization, Cell division, Cysteine-rich, TCR motif, *Arabidopsis thaliana*

INTRODUCTION

The shoot apical meristem (SAM) is the source of all aerial parts of plants. In *Arabidopsis*, a self-perpetuating stem cell system in the SAM is responsible for the repetitive formation of leaves and secondary shoots during vegetative development, and the same system is responsible for the repetitive generation of flowers during later reproductive development. This later SAM that develops flowers instead of shoots is termed 'inflorescence meristem' (IM), in which flowers are initiated at the periphery in a spiral pattern (Fig, 1A,B). A meristem (whether SAM or IM) can be divided into three zones: the central zone at the apex of the meristem where cell division is infrequent, the peripheral zone that surrounds the central zone where cell divisions are relatively rapid, and the rib meristem (or rib zone) beneath the central zone, where divisions are also rapid (Fig. 1C; Steeves and Sussex, 1989; Meyerowitz, 1997; Clark, 1997). The central zone harbors the stem cells that divide slowly to generate daughter cells to the surrounding peripheral zone, where these daughter cells are incorporated into differentiating leaf, shoot or flower primordia.

The *Arabidopsis CLAVATA* genes *CLV1*, *CLV2* and *CLV3* regulate a critical aspect of SAM development. Loss-of-function *clv* mutations cause progressive SAM enlargement due to accumulation of undifferentiated cells in the central zones (Clark et al., 1993, 1995; Kayes and Clark, 1998; Fletcher et al., 1999). Thus, *CLV* genes either function to repress cell division in the central zone or to promote the transition of cells from an undifferentiated state toward organ formation and differentiation. *CLV1* encodes a putative receptor kinase, while *CLV3* encodes the putative ligand for the *CLV1* receptor (Clark et al., 1997; Fletcher et al., 1999). CLV2, also a receptor-like protein, functions in the same pathway as *CLV1* and *CLV3* in regulating meristem development (Kayes and Clark, 1998; Jeong et al., 1999). *SHOOT MERISTEMLESS* (*STM*) from *Arabidopsis* encodes a homolog of maize homeobox gene *KNOTTED1* (*KN1*) (Vollbrecht et al., 1991; Long et al., 1996) and acts to oppose the function of *CLV* (Clark et al., 1996; Endrizzi et al., 1996). These and other molecular genetic studies are beginning to reveal the molecular mechanism for SAM formation and maintenance.

Another important aspect of meristem development is the regulation and execution of basic cell division processes. Since plant cells are surrounded by a rigid wall, the development and maintenance of the meristem requires regulated cell divisions with respect to the rate and orientation of division. For example, meristems in most flowering plants can be divided into three layers, L1, L2 and L3 (Fig. 1C; Poethig, 1989; Kerstetter and

Hake, 1997; Meyerowitz, 1997). Cells in the L1 and L2 divide anticlinally (with the new division plane perpendicular to the surface) and cells in L3 divide in all directions. Cells in the L1 layer give rise to epidermis, the L2 layer to subdermal tissues, and the L3 layer to core tissues. Thus, cell division regulation plays an important role in maintaining meristem cell layers and subsequently cell fate determination. In *Arabidopsis*, mutations that affect basic processes of cell division have been identified. Mutations in *KNOLLE* (*KN*) (Lukowitz et al., 1996), *KEULE* (*KEU*) (Jurgens et al., 1994; Assaad et al., 1996), *CYT1* (Nickle and Meinke, 1998), and *GNOM/EMB30* (Mayer et al., 1991; Shevell et al., 1994) all affect basic cell division processes and exhibit embryonic lethality. *kn, keu* and *cyt1* mutant cells exhibit incomplete cytokinesis with crossed walls (or wall stubs) and multinucleated cells. *KN* encodes a protein with similarity to syntaxin, suggesting that *KN* may be involved in the transport of vesicles for cell plate formation (Lukowitz et al., 1996). *GNOM/EMB30* encodes a protein with similarity to the yeast *SEC 7* gene in the secretary pathway for vesicle budding (Shevell et al., 1994). Thus the genetic identification and subsequent isolation of these *Arabidopsis* genes are beginning to shed light on the plant cell division mechanism and the developmental consequences.

Here, we report the molecular analysis of another *Arabidopsis* gene *TSO1* that plays an important role in both meristem organization and basic cell division processes. Previously, our analyses of *tso1* mutants were focused on the cell division defects (Liu et al., 1997). In cells of *tso1* mutant flowers, nuclei were of irregular size and shape and possessed increased DNA content. In addition, these cells frequently exhibited partially formed cell walls, highly invaginated nuclear membranes, and multinucleated cells. Furthermore, the L1 and L2 cell layers were not well maintained. Nevertheless, the cell division defects were only observed in cells of the floral meristem and floral organs (Fig. 1E; Liu et al., 1997). A second and less understood developmental defect is the frequent fasciation in the inflorescence meristems of *tso1-1* mutants, resulting in thick and flat stems and compound inflorescences (Fig. 1E). This defect is further analyzed in this study. Finally, two types of *tso1* alleles have been isolated that exhibit superficially very different phenotypes. While both *tso1-1* and *tso1-2* display strong phenotypes described above, a third allele, *tso1-3*, exhibits defects only in ovules (Hauser et al., 1998). The integument cells of the *tso1-3* mutant ovules have abnormal shape and alignment, and the embryo sac is absent due to an inability to form the megaspore mother cells. The flowers of *tso1-3* are normal with only slightly ragged shape at the tip of sepals and petals (Hauser et al., 1998; Fig. 1D). Nevertheless, the *tso1-3* phenotype results from a defect in the same gene as *tso1-1* (Hauser et al., 1998).

To understand the molecular basis of the various defects of *tso1* mutants, we used a map-based approach to isolate the *TSO1* gene. We report that *TSO1* encodes a novel protein with conserved cysteine (cys)-rich repeats. *TSO1* mRNA is expressed in the floral meristem and floral organs during active cell division. TSO1-GFP chimeric proteins are localized to the nucleus. Based on the molecular analyses of the *TSO1* gene and additional phenotypic characterization of the inflorescence meristem defects in *tso1* mutants, we propose a model that explains the role of *TSO1* in regulating meristem development and floral cell division.

MATERIALS AND METHODS

Plant growth, mutant strains and microscopy

Plant growth conditions were described previously (Liu and Meyerowitz, 1995). The *tso1-1* and *tso1-2* mutations were isolated in two different EMS mutagenesis screens and were described by Liu et al. (1997) and Levin et al. (1998) respectively. *tso1-3* is a gift from Drs B. Hauser and C. Gasser (Hauser et al., 1998). All three alleles were generated in the Landsberg *erecta* (L*er*) ecotype.

For scanning electron microscopy (SEM), inflorescences were fixed, coated, dissected and photographed as described previously (Bowman et al., 1989, 1991). Images were directly captured with the semicaps software and the AMRAY 1000A scanning electron microscope. The 4′, 6′diamidino-2-phenylindole (DAPI) staining and light microscopy of tissue sections were based on the method of Friedman (1991).

Map-based cloning

TSO1 was first mapped to chromosome 3 between molecular markers *nga162* (Bell and Ecker, 1994) and *SUPERMAN* (*SUP*; Sakai et al., 1995) at 16 and 6 map units respectively. Using these two markers, additional recombinants between L*er* and Columbia (Col) ecotypes were identified by screening 219 individual F_3 *tso1-1* mutants. Analyses of these recombinants further mapped *TSO1* between markers PAP606 and T27F6R. This region is covered by the YAC CIC7B12 and two overlapping BACs T27F6 and T18B19 (Fig. 2). Cosmid libraries were constructed from the BAC T27F6 and YAC CIC7B12 respectively using the binary vector pCLD04541. The procedure is essentially the same as described by Bent et al. (1994). Cosmid clones were isolated using T27F6, mi268, PAP606 and 27F6R as probes. Restriction mapping and Southern analyses enabled the construction of a cosmid contig covering the entire 80 kb region of the BAC T27F6 (Fig. 2).

Because *tso1-1/tso1-1* mutants are completely sterile, *sup-5*, a weak and fertile *sup* allele (Gaiser et al., 1995) is used to balance the *tso1-1* mutation. *tso1-1+/+sup-5 trans*-heterozygous plants were infected with *Agrobacterium* strain GV3101 carrying corresponding cosmids. Seven overlapping cosmids were transformed into *tso1-1+/+sup-5 trans*-heterozygous plants using the vacuum-infiltration method (Bechtold et al., 1993). Primary transgenic plants were selected from independent transformations. If a cosmid contained the *TSO1* gene, the number of *tso1-1* mutants in primary transgenic plants would be greatly reduced (much lower than the 25%). Two overlapping cosmids: #56 and #8 fulfilled such a criterion. Only 3.8% (1/26; #56) and 1.9% (3/156; #8) of the primary transgenic plants exhibited a *tso1-1* phenotype. The progeny of the #56 primary transformants were analyzed further by PCR. Co-segregation of cosmid #56 with the suppressed phenotype is 100% (24/24). Cosmid #36 overlaps with cosmid #56 and #8 and partially rescued the *tso1-1* phenotype (Figs 1H, 2).

Isolation and analyses of the *TSO1* transcript

Approximately 1 million plaques of an *Arabidopsis* flower cDNA library (a gift from Dr Detlef Weigel) were screened. Five *TSO1* cDNA clones were isolated. For northern analyses, poly(A)$^+$ RNA was isolated from respective tissues using the FastTrack 2.0 RNA isolation system (Invitrogen). Rosette leaves, cauline leaves and stems were harvested from the same mature flowering plants. Seedlings were harvested at approximately 7 days after germination when they had developed two to four leaves. Roots were cut off from 2-week old seedlings that were growing vertically in a petri dish. Floral tissues were isolated from inflorescences that contain flowers of all stages. 3-4 μg poly(A)$^+$ RNA was loaded per lane and fractionated in a 0.9% agarose gel according to Sambrook et al. (1989). Northern filters were probed with a gene-specific *TSO1* probe derived from a *Xho*I fragment (592-1028 bp of *TSO1* cDNA; Fig. 3). The 18S RNA probe was derived from an *Arabidopsis* EST 246D24T7. The actin probe is a 1.6

kb actin cDNA from *Brassica oleracea* (Stein et al., 1991). All hybridizations were carried out in 6× SSPE, 5× Denhardt's solution and 0.5% SDS at 65°C. The final washes of the membrane were at 65°C in 0.2× SSPE and 0.1% SDS.

A 0.4 kb DNA fragment corresponding to 424-824 bp of the *TSO1* cDNA (Fig. 3) was cloned into the pSK vector (Stratagene) and served as a gene-specific probe for *in situ* hybridization. To generate a *TSO1* antisense RNA probe, the plasmid was linearized with *Eco*RI and used as a template for in vitro transcription in the presence of T7 RNA polymerase (Epicentre) and digoxigenin (DIG)-UTP (Boehringer Mannheim). Tissue fixation and hybridization procedures were described previously (Liu and Meyerowitz, 1995).

5′RACE was conducted using the 5′Race kit version 2.0 (GIBCO-BRL). Three nested primers from the 5′ gene-specific region of *TSO1* cDNA were used: 5′-CACTGATGTTACTGTCCTTTGC-3′, 5′-TTGTAGGAAGCACAGCCAAAGG-3′, and 5′-CCAAGCATCTAA-AAGCCTCAGA-3′. Two prominent bands of size 820 bp and 320 bp were produced after 5′ RACE PCR. Both bands were purified and then sequenced (ABI DNA sequencer 373A).

GFP-TSO1 chimeric gene construction and transient expression

Full length *TSO1* cDNA was PCR-amplified with primers 5′ AGCCATGGACAAATCCCAGAAGA 3′ and 5′ TCCATGGGG-TTGAGAGAAGGAAAT 3′ using the Expand^{TM} High Fidelity PCR System (Boehringer Mannheim) and a full length *TSO1* cDNA clone as the template. The amplified fragment was cloned into the *Nco*I restriction site of the vectors pAVA120 (von Arnim et al., 1998). The resulting plasmid construct, verified by sequence analyses, carries an in-frame fusion of full length TSO1 to the NH_2 terminus of GFP. In a second construct, a partial *TSO1* cDNA from 638 bp to the stop codon (Fig. 3) was PCR-amplified with primers 5′ AGATCT-CAGAATATGCTTGTTGTTC 3′ and 5′ AGATCTGCTAATAGG-ATCTGGAACA 3′. The amplified fragment was cloned into pCR2.1 (TA cloning kit; Invitrogen) and sequenced. The *Bgl*II fragment of the pCR2.1-partial TSO1 was inserted into the pAVA120 vector (von Arnim et al., 1998), resulting an in-frame fusion of TSO1 to the COOH terminus of GFP. Transient expression assays in onion epidermal cells were carried out using a Biolistic Particle Delivery System (Bio-Rad) according to the method of Sanford et al. (1993). Typically, 2-5 μg of either pAVA120 or pAVA120-TSO1 plasmid was used. The tissues were visualized under a Nikon Labophot-2 microscope equipped with a FITC filter. Images were obtained with a 20× objective and a Nikon 2000 35 mm camera.

RESULTS

tso1 fasciation results from bifurcation or multiple splitting of inflorescence meristems

In vegetative stage, the SAMs of strong *tso1-1* and *tso1-2* mutants are slightly abnormal. The *tso1* mutant SAM develops, on average, 6 secondary shoots (i.e. axillary shoots) (n=28), while wild-type SAM develops 3-4 secondary shoots. In addition, *tso1* plants appear bushier and form higher order axillary shoots (from second to sixth order), probably due to a slight loss of apical dominance.

When the strong *tso1* plants enter reproductive phase, almost all of their inflorescence meristems develop thick stems and compound inflorescences with many more flowers (Fig. 1E). This fasciation results from extensive bifurcation, or multiple splitting of the inflorescence meristems (Fig. 1E-G). Each inflorescence is first split into two, three or more inflorescences, which are then split into two or more secondary inflorescences. Thus, in contrast to *clv* mutants,

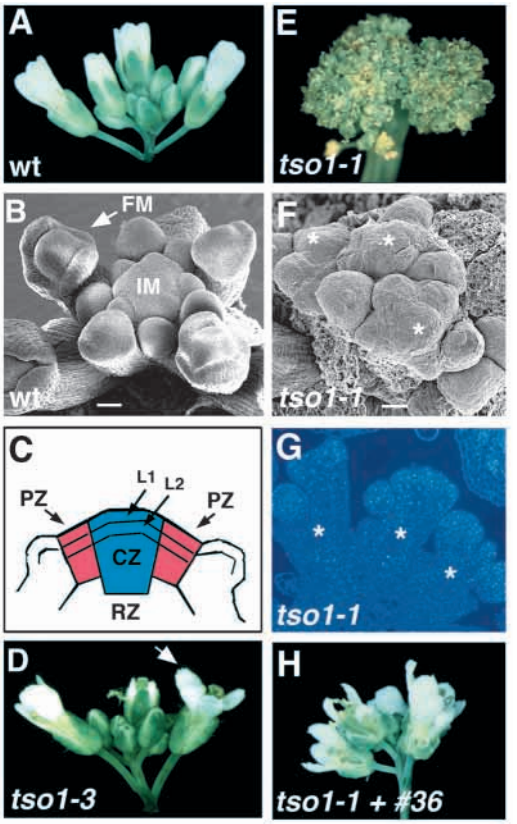

Fig. 1. Wild-type and *tso1* inflorescences. (A) A wild-type inflorescence. (B) A scanning electron microscopic (SEM) image of a wild-type inflorescence. Floral meristems (FM) are being generated from the inflorescence meristem (IM) in a spiral pattern. (C) A diagram of a wild-type inflorescence meristem showing the central zone (CZ; blue), peripheral zone (PZ; red) and rib zone (RZ). L1 and L2 cell layers are also indicated. (D) A *tso1-3* inflorescence. Flowers are similar to wild-type with the exception of rough petal edges (arrow) and sepal tips (Hauser et al., 1998). (E) A *tso1-1* inflorescence. Extensive fasciation results in an enlarged inflorescence and the formation of many more abnormal flowers. Each flower only develops abnormal sepals (Liu et al., 1997). (F) A SEM image of a *tso1-1* mutant shoot apex. Three inflorescence meristems are formed on the same shoot apex; each is indicated by an asterisk. (G) A 1μm thin longitudinal section of a *tso1-1* inflorescence shoot. Tissues were stained with the DNA staining dye DAPI. Three inflorescences are each indicated by an asterisk. All of them are derived from a single inflorescence. (H) An inflorescence of a second generation *tso1-1* transgenic plant containing cosmid #36. All four types of floral organs were present, but sepals and petals exhibited whitish edges, and the flowers were sterile. Bar in B and F, 20 μm.

fasciation in strong *tso1* mutants is not caused by a single enlarged inflorescence meristem, but rather by the formation of multiple inflorescence meristems. Moreover, *tso1* fasciation appears to be largely specific to the inflorescence meristem, when the SAM starts to develop flowers instead of shoots.

Finally, floral meristems are formed at the periphery of the inflorescence meristem (Fig. 1B) and give rise to four types of floral organs in four concentric whorls. Unlike inflorescence meristems, floral meristems in strong *tso1* mutants do not exhibit any splitting from one floral meristem into multiple

floral meristems. However, the number of sepals are frequently increased from 4 in wild-type to 5 or 6 in the *tso1-1* mutants (Liu et al., 1997).

Positional cloning of *TSO1*

We isolated the *TSO1* gene using a map-based approach. *TSO1* was first mapped to chromosome 3 between two molecular markers, *nga162* and *SUPERMAN (SUP)* (Liu et al., 1997). *nga162* and *SUP* were used to screen a mapping population for recombinants. Analyses of these recombinants subsequently narrowed *TSO1* to a single yeast artificial chromosome (YAC) that was also covered by two overlapping bacterial artificial chromosomes (BACs) (Fig. 2). Cosmid libraries were constructed from the YAC and the BAC (T27F6), and a cosmid contig was constructed covering an 80 kb region (Fig. 2). Cosmids in the contig were tested for their ability to rescue the *tso1-1* mutant. We found that two overlapping cosmids #56 and #8 completely rescued the *tso1-1* mutants, while another overlapping cosmid #36 partially rescued the *tso1-1* mutant (Fig. 1H, 2; Materials and Methods). The *TSO1* gene therefore resides in the overlapping region of these three cosmids.

TSO1 encodes a protein with cysteine-rich repeats

cDNA clones were isolated from an *Arabidopsis* flower cDNA library using cosmid #56 as a probe. These cDNA clones define three different genes, one of which is located in the overlapping region of cosmid #36, #8 and #56 (Fig. 2). Sequence analyses of this cDNA revealed at least two motifs: a nuclear localization signal and two cysteine-rich (cys-rich) repeats (Fig. 3). Sequence analyses of genomic DNA in this gene revealed single base substitutions in each of the three *tso1* alleles (Fig. 3). *tso1-1* contains a G to A transition that results in a cysteine to tyrosine substitution in the second cys-rich repeat (Fig. 3). Similarly, *tso1-2* causes a G to A transition that results in a different cysteine to tyrosine substitution in the first cys-rich repeat (Fig. 3). *tso1-3*, a weak *tso1* allele, contains a nonsense mutation; a C to T change results in an early termination of translation and deletes one of the two cys-rich repeats. It is unclear why removal of one of the two cys-rich motifs in *tso1-3* is less detrimental than a single cysteine substitution in the cys-rich region in *tso1-1* or *tso1-2*. Nevertheless, both the cosmid rescue and the identification of mutations in all *tso1* alleles lead us to conclude that we have isolated the *TSO1* gene.

Database searches reveal a class of plant genes that possesses similar cys-rich repeats as TSO1 (Fig. 4A,B). For convenience, we designate this cys-rich repeat motif as 'TSO1 Cysteine-Rich' motif or TCR motif. All genes in this class possess two copies of the TCR motif (Fig. 4A,B) and a 54 amino acid intervening region between the two TCR motifs. This class of genes includes *TSO1* and several *Arabidopsis* genes with unknown function. They are *TSO1-like* (Z97337), two hypothetical proteins (AC009465 and AC006081), and a '*Putative Transcription Factor*' (PTF) (AL078470). Among them the TSO1-like and the hypothetical protein AC009465 are similar to TSO1 throughout the entire coding region with 47% and 28% identity respectively. However, in the TCR repeat 1 of these two genes, the first 13 amino acid are not similar to TSO1 (Fig. 4A). The other member of this class is a soybean gene encoding the *Cysteine-rich Polycomb-like Protein 1* (*CPP1*; AJ010165). CPP1 is a regulator of the *LEGHEMOGLOBIN* gene expression (E. Ø. Jensen, personal communication) and exhibits a high level of sequence similarity (56% identity) to TSO1 in the TCR repeats and the intervening region between the two repeats. However, the similarity between CPP1 and TSO1 is significantly reduced outside this region.

The *Drosophila Enhancer of zeste (E(z))* and its plant homologs are members of a second class of genes that share sequence similarity with *TSO1* (Fig. 4C). *Drosophila* E(z) is a member of the polycomb group proteins (Jones and Gelbart, 1990); polycomb group proteins ensure the stable inheritance of expression patterns through cell division and regulate the control of cell proliferation (Pirrotta, 1998). At the COOH terminus, E(z) contains a SET domain, named after the three founding fathers of the family in *Drosophila* (Jenuwein et al., 1998). Unique to E(z) is the presence of a CXC domain, a cys-rich region (aa. 538-603) preceding the SET domain. Only a portion of this CXC domain (aa. 573-603) is similar to the TCR motif (Fig. 4C). This portion of the CXC domain of several *Arabidopsis* E(z) homologs is similar to the TCR motif (Fig. 4C). These *Arabidopsis* E(z) homologs include *CLF-Like* (AF001308), *EZA1* (AF100163), *Polycomb-Group (POLYC-G;* Y10580), *CURLY LEAF* (*CLF*; Goodrich et al., 1997), and *MEDEA* (*MEA*, Grossniklaus et al., 1998; Kiyosue et al., 1999). Of these genes, the functions of *CLF* and *MEA* are

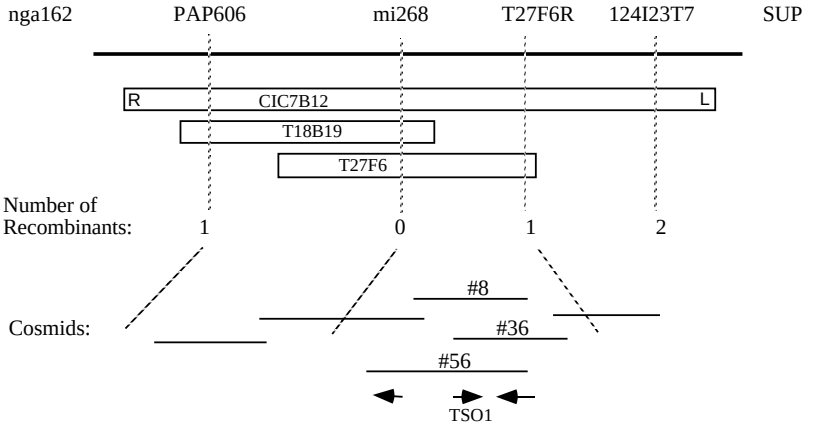

Fig. 2. Physical map of the *TSO1* region. *TSO1* maps to chromosome 3 between *nga162* and *SUP* at 16 and 6 map units respectively. The number of recombinants indicates the relative distance between *TSO1* and the corresponding molecular markers, PAP606, mi268, T27F6R, and 124I23T7. *TSO1* is closely linked to mi268 as shown by zero recombinant. The YAC clone CIC7B12 and the BAC clones T18B19 and T27F6 are shown as open bars. Cosmid clones are represented by solid lines. Each cosmid is about 20 kb in insert size. Only the most relevant cosmids are shown. Cosmids #56 and #8 completely rescued the *tso1-1* mutant phenotype, while cosmid #36 partially rescued the *tso1-1* mutant phenotype (Fig. 1H). Cosmid #36 starts at the first intron of *TSO1* and likely utilizes the second transcription initiation site or transcription read-though from vector sequences. Three genes are present in cosmid #56 as indicated by lines with arrows. The arrow points to the direction of transcription.

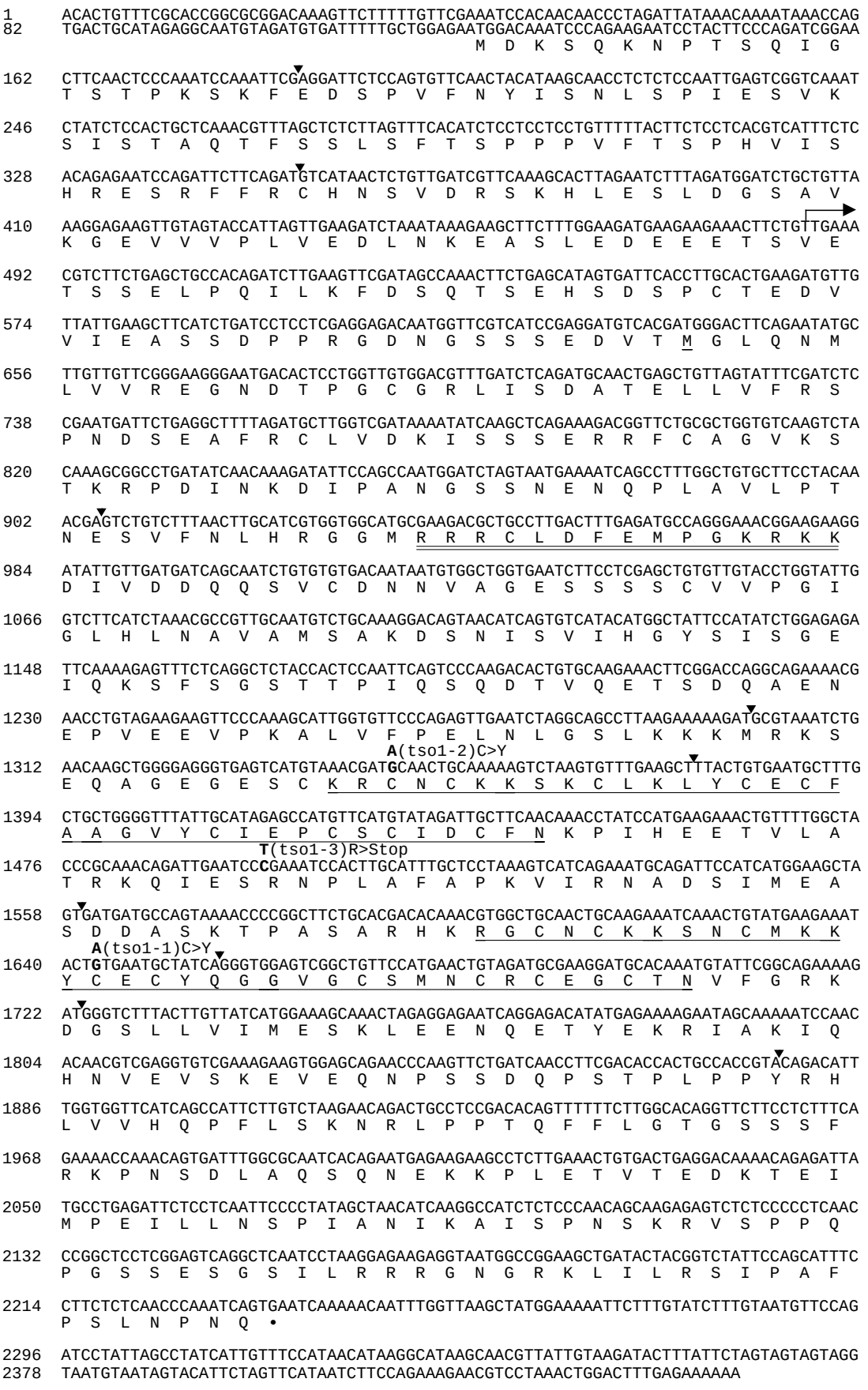

Fig. 3. Sequence of *TSO1* cDNA and the deduced amino acids. Intron positions are indicated by arrowheads. A nuclear localization signal is doubly underlined. The TCR repeats are underlined. Base pair changes found in all three *tso1* alleles are indicated above the wild-type sequence. The second transcription initiation site for the 2.0 kb transcript is indicated by an angled arrow in the third exon. The second methionine is underlined and may serve as the translation initiation codon for the 2.0 kb transcript. The *TSO1* cDNA sequence has been submitted to GenBank (accession number, AF206324).

(A) TCR Repeat 1

TSO1	401	K R **C** N C K K S K C L K L Y C E C F A A G V Y C I E P C S C I D C F N
TSO-LIKE	359	L Q E L N L S S P K K K S Y C E C F A A G V Y C I E P C S C I D C F N
AC009465	415	G E C F D Q L M A M E N R Y C E C F S A G L F C G E P C S C Q N C F N
CPP1	474	K R C N C K K S K C L K L Y C D C F A A G T Y C T D P C A C Q G C L N
PTF	132	K Q C N C K H S R C L K L Y C E C F A S G T Y C D G – C N C V N C F N
AC006081	119	K Q C N C K H S R C L K L Y C E C F A S G T Y C D G – C N C V N C F N

(B) TCR Repeat 2

TSO1	487	R G C N C K K S N C M K K Y **C** E C Y Q G G V G C S M N C R C E G C T N (X)$_5$ D G S L
TSO1-LIKE	445	R G C N C K K S N C L K K Y C E C Y Q G G V G C S I N C R C E G C K N (X)$_5$ D G S S
AC009465	501	R G C N C R K S G C S K K Y C E C F M M G V G C S S N C R C M G C K N (X)$_5$ N E Q C
CPP1	560	R G C N C K R S M C L K K Y C E C Y Q A N V G C S S G C R C E G C K N (X)$_5$ E D Y V
PTF	217	K G C H C K K S G C L K K Y C E C F Q A N I L C S E N C K C L D C K N (X)$_5$ E R Q A
AC006081	204	K G C H C K K S G C L K K Y C E C F Q A N I L C S E N C K C L D C K N (X)$_5$ V R Q S

(C) A Portion of the CXC Domain in E(z) Family

CLF-LIKE	687	R G C H C A K S Q C R S R Q C P C F A A G R E C D P D V – C R N C W V (X)$_3$ D G S L
EZA1	645	R G C H C A K S Q C R S R Q C P C F A A G R E C D P D V – C R N C W V (X)$_3$ D G S L
POLYC-G	689	R G C H C A K S Q C R S R Q C P C F A A D R E C D P D V – C R N C W V (X)$_3$ D G S L
CLF	689	R G C H C A K S Q C R S R Q C P C F A A D R E C D P D V – C R N C W V (X)$_3$ D G S L
MEA	484	G G C N C A I G Q C T N R Q C P C F A A N R E C D P D L – C R S C P L (X)$_3$ D G I L
E (Z)	573	P G C R C – K A Q C N T K Q C P C Y L A V R E C D P D L – C Q A **C** G A (X)$_5$ T K I T

Fig. 4. Sequence alignment in the TCR motif. Two classes of genes share sequence similarity in the TCR motif. The first class contains two copies of the TCR motif presented in A and B respectively. The second class is represented by the *Drosophila* E(z) and its plant homologs shown in C. Conserved cysteine residues are boxed. (A) Alignment of the first TCR repeat in TSO1, TSO1-LIKE (Z97337), hypothetical protein AC009465, Cysteine-rich Polycomb-like Protein (CPP1) (AJ010165), Putative Transcription Factor (PTF) (AL078470), and the hypothetical protein AC006081. The bold and underlined **C** is mutated in *tso1-2*. (B) The second TCR motif in the same group of proteins listed in A. The bold and underlined **C** is mutated in *tso1-1*. TSO1 possesses the **DGSL** following the TCR repeat 2. (C) A portion of the CXC domain of *Drosophila* E(z) (Jones and Gelbart, 1993) and its *Arabidopsis* homologs: CLF-Like (AF001308), EZA1 (AF100163), Polycomb-Group (POLYC-G) (Y10580), CURLY LEAF (CLF; Goodrich et al., 1997), and MEDEA (MEA; Grossniklaus et al., 1998; Kiyosue et al., 1999). The bold and underlined **C** is mutated in E(z)^{61}. With the exception of E(z), the last four amino acids **DGSL** are found in all members of this class. TSO1 is the only protein in the first class that possesses this **DGSL** (B).

known. *CLF* encodes a transcriptional repressor of floral homeotic gene *AGAMOUS* (Goodrich et al., 1997), and *MEA* functions as a suppressor of endosperm development (Kiyosue et al., 1999). Of particular interest is the presence of 4 amino acids 'DGSL' following the TCR repeat 2 of TSO1 (Fig. 4B). This DGSL is also found at similar positions in CLF-LIKE, EZA1, POLYC-G, CLF and MEA (Fig. 4C). Obviously, the similarity between TSO1 and genes of E(z) class is only limited to the TCR/CXC motif.

As illustrated in Fig. 4, the position and the spacing of the cysteine residues are highly conserved, indicating the importance of these cysteine residues. The *tso1-1*, *tso1-2* and *E(z)*^{61} mutations all possess an amino acid substitution, replacing one of these highly conserved cysteine residue with a tyrosine residue (Fig. 4; Carrington and Jones, 1996). The strong defects associated with mutational substitutions of

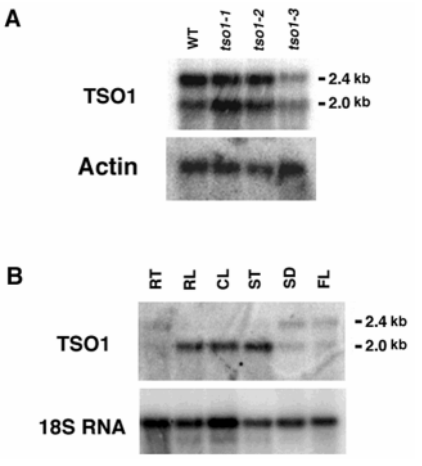

Fig. 5. *TSO1* mRNA expression in different tissues. (A) Northern analyses of mRNAs isolated from inflorescences of wild-type and three *tso1* mutants. Two *TSO1* transcripts were detected with a gene-specific *TSO1* probe (see Materials and Methods). Based on size markers, the top band is estimated at 2.4 kb and the lower band is estimated at 2.0 kb. These two bands are likely due to transcriptional initiation from two different sites (Fig. 3). Equal amount of poly (A)$^+$ RNA was loaded in each lane as shown by the amount of actin transcript. (B) Northern analyses of *TSO1* mRNA isolated from wild-type roots (RT), rosette leaves (RL), cauline leaves (CL), stems (ST), seedlings (SD) and inflorescences (FL). The same gene-specific *TSO1* probe was used. Specific stages of these tissues are described in Materials and Methods. The 18S RNA band serves as a loading control.

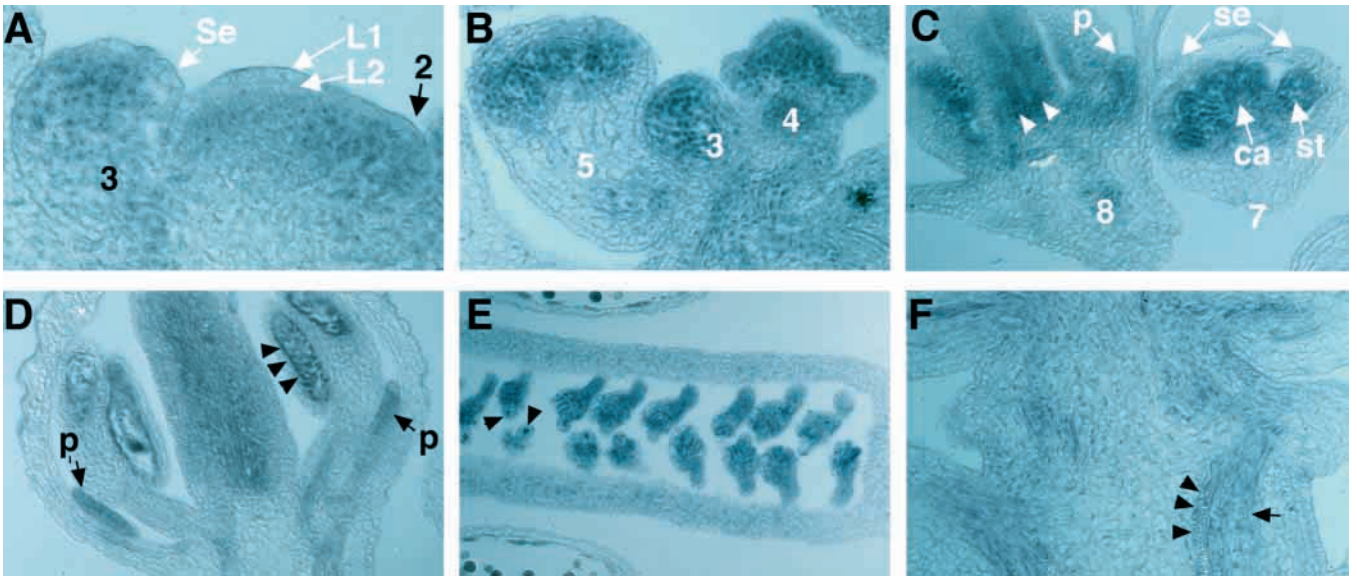

Fig. 6. *TSO1* mRNA is expressed in actively dividing cells. 8 μm longitudinal sections of wild-type *Arabidopsis* inflorescence apices were hybridized with a DIG-labeled, gene-specific *TSO1* antisense RNA probe (see Materials and Methods). Numbers indicate the stage of flowers, according to Smyth et al. (1990). (A) A wild-type inflorescence meristem flanked by a stage 2 and a stage 3 floral meristem. *TSO1* mRNA was detected in most cells of the stage 2 floral meristem with the exception of epidermal cells (L1 cells). In the stage 3 flower, *TSO1* expression was stronger in the center of the flower, the developing sepals exhibited a reduced level of *TSO1* mRNA. In the inflorescence meristem, *TSO1* mRNA was barely detectable in the L1 layer and was also absent from a few L2 layer cells in the center. (B) Three flowers at different stages of development. *TSO1* mRNA was expressed in the entire floral meristem of an early stage 3 flower. The stage 4 flower on the right strongly expressed *TSO1* in the center. *TSO1* mRNA was diminishing from the abaxial side of the sepals. *TSO1* was expressed in the developing stamens and carpels and the adaxial surface of sepals in the stage 5 flower. Petals are not seen at these stages. (C) In a stage 7 flower, *TSO1* mRNA was not detectable in sepals (se), but was expressed at a high level in the stamen and carpel primordia. In the stage 8 flower, *TSO1* mRNA in carpels was largely restricted to the placental region (arrowheads). The expression of *TSO1* in petals (p) was evident. (D) *TSO1* mRNA was detected in developing petals (p) of a maturing (stage 10) flower. *TSO1* was highly expressed in the single layer of tapetum cells (arrowheads) of the anther. The tapetum cell layer encloses and provides nutrition for developing pollen. These developing pollen (microspores) appeared to express *TSO1* as well. (E) Developing ovules within the gynoecium of a mid-stage 12 flower. *TSO1* was highly expressed in the inner and outer integuments and in the funiculus. *TSO1* mRNA was also detected in the 4-nucleate haploid embryo sac (arrowheads). *TSO1* mRNA was not detected in the carpel walls. (F)*TSO1* RNA was detected in the provascular tissues adjacent to the xylem (arrow). Three arrowheads indicate xylem.

cysteine residues in *tso1-1*, *tso1-2* and $E(z)^{61}$ indicate that the TCR motif encodes an important functional domain.

Two *TSO1* transcripts are expressed in different tissues

Two *TSO1* transcripts of different sizes were detected by 5′ RACE (See Materials and Methods) and by northern analyses (Fig. 5). Sequence analyses of 5′ RACE PCR products revealed that the two *TSO1* transcripts result from different transcriptional initiation sites spaced 400 bp apart (Fig. 3). The longer *TSO1* transcript encodes a putative protein of 695 amino acids, while the shorter transcript initiates from the third exon and encodes a putative protein of 526 amino acids (Fig. 3). In northern analyses (Fig. 5A), the two transcripts were detected by a gene-specific *TSO1* probe (see Materials and Methods). One transcript is estimated at 2.4 kb while the other is at 2.0 kb. Only the 2.4 kb transcript was detected with a 5′ *TSO1* probe (data not shown).

TSO1 mRNA expression in floral tissue was examined in wild-type and all three *tso1* mutants. *TSO1* mRNA is not expressed at a high level in flowers and can only be detected in RNA blots using poly(A)$^+$ RNA. Both *TSO1* transcripts in *tso1-3* mutants were at a reduced level (40% of the wild-type level) (Fig. 5A). This reduction suggests that the premature translational termination caused by the *tso1-3* mutation may render its mRNA unstable. In *tso1-1* and *tso1-2* mutants, the

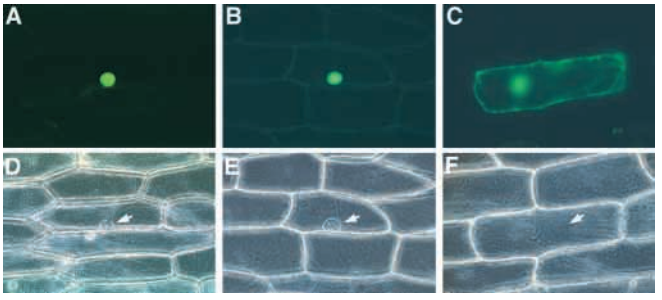

Fig. 7. Nuclear localization of TSO1-GFP. (A-C) Dark-field images; (D-F) Corresponding phase-contrast microscopic images. Arrows mark the location of nucleus. (A,D) An onion epidermal cell transiently expressing the full length TSO1 (695 amino acids) fused to the NH_2 terminus of GFP. TSO1-GFP was only detected in the nucleus. (B,E) An onion epidermal cell transiently expressing a smaller TSO1 protein (526 amino acids) fused to the COOH terminus of GFP. Similarly, this GFP-TSO1 was only detected in the nucleus. (C,F) An onion epidermal cell transiently expressing the GFP protein. GFP was found in both cytoplasm and nucleus.

2.4 kb transcript was expressed at a level similar to that of wild-type. However, the 2.0 kb transcript appeared more abundant in these two mutants than in wild-type (Fig. 5A). Densitometry analyses revealed that, in wild-type, the 2.0 kb transcript was only half (50%) the amount of the 2.4 kb transcript, but in *tso1-1* and *tso1-2* mutants, the 2.0 kb transcript was 130% and 83% the amount of the 2.4 kb transcript respectively. The increased level of the 2.0 kb transcript in *tso1-1* and *tso1-2* mutants (Fig. 5A) might result from the absence of the inner three whorls of floral tissues in the mutant flowers where the 2.4 kb transcript might be better represented. Alternatively, the increased level of the 2.0 kb transcript in *tso1-1* and *tso1-2* mutants could reflect an autoregulatory role of *TSO1*.

Since the flower- and inflorescence-specific phenotype of *tso1* might result from tissue-specific transcriptional regulation, *TSO1* mRNA expression was examined in different tissues. The 2.0 kb *TSO1* mRNA was detected in all tissues examined (Fig. 5B) including 2-week old roots, 7-day old seedlings, mature cauline leaves, rosette leaves, stems, and flowers (in a inflorescence). Interestingly, in rosette leaves, cauline leaves and stems, this 2.0 kb transcript was expressed at a much higher level than in roots, seedlings and the flowers. In contrast, the 2.4 kb *TSO1* mRNA was only detected in roots, seedlings and flowers and was not detected in the leaves and stems (Fig. 5B). Thus, the two *TSO1* transcripts are under different regulation in different tissues.

TSO1 mRNA is abundant in actively dividing cells in flowers

A *TSO1* gene-specific probe that detects both transcripts was used to examine *TSO1* mRNA distribution by in situ hybridization. *TSO1* expression was weakly detected in the inflorescence meristem in an interesting pattern (Fig. 6A). First, it appeared absent from the epidermal (L1) layer of the inflorescence meristem. This absence of *TSO1* mRNA in epidermis appeared to extend to the peripheral zone and even stage 1 floral meristems. Second, *TSO1* mRNA was absent in a small number of cells (about 2-3 cells) in the L2 layer within the central zone but was present in the L2 layer in the peripheral zone. This absent or reduced *TSO1* mRNA expression appeared to coincide with the upper half of the central zone in inflorescence meristems.

In flowers, *TSO1* mRNA was expressed in a dynamic pattern. At stage 1 and stage 2, with the exception of the epidermis, *TSO1* mRNA was detected in the entire floral meristem (Fig. 6A). At late stage 3 and onward, *TSO1* mRNA was restricted to the center of the flower and the expression subsided from sepal primordia initially from the abaxial side (Fig. 6A-C). Later, *TSO1* mRNA was detected in developing petals, stamens and carpels (Fig. 6B-D). Thus, it appears that *TSO1* is expressed in young floral meristems or developing floral organs when cells are actively dividing. When floral organ growth was completed, *TSO1* mRNA was detected only in male and female reproductive tissues. Specifically, a high level of *TSO1* mRNA was detected in the locules of anthers during microsporogenesis, in the tapetum and the microspores (Fig. 6D). *TSO1* mRNA was also present in developing ovules during megasporogenesis: early in the entire ovule primordia (data not shown) and later in funiculus, integuments, and the embryo sac (Fig. 6E). Thus *TSO1* may function in both diploid and haploid cells undergoing active cell division. Consistent with our northern results, *TSO1* mRNA was detected in the stem, in what appeared to be vascular cambium and procambium cells of the stem (Fig. 6F).

TSO1-GFP fusion proteins localize to the nucleus

We examined the subcellular location of the TSO1 protein using a Green Fluorescent Protein (GFP) tag. Two types of TSO1-GFP fusion proteins were made (see Materials and Methods). First, full length TSO1 protein was fused in-frame to the NH_2 terminus of GFP. Second, a partial TSO1 protein (from the second methionine to stop; Fig. 3) was fused in-frame to the COOH terminus of GFP. This smaller TSO1 protein is a putative translational product of the 2.0 kb *TSO1* transcript. Vector pAVA120 (von Arnim et al., 1998) was used in both of these constructs, in which the GFP-TSO1 chimeric genes are under the transcriptional control of the CaMV 35S promoter. These GFP-TSO1 constructs were transiently transformed into the onion epidermal cells by particle bombardment (Sanford et al., 1993). In all cases, the green fluorescent TSO1-GFP proteins were detected in the nucleus only (Fig. 7A,B,D,E), while the control construct of GFP alone was detected in cytoplasm and nucleus (Fig. 7C,F). This nuclear localization of TSO1-GFP is consistent with a role of TSO1 in transcriptional regulation.

DISCUSSION

TSO1 encodes a protein with cys-rich repeats implicated for DNA binding

We show that *TSO1* encodes a novel protein with two cys-rich repeats (or TCR repeats). Several pieces of evidence indicate the importance of this TCR motif for *TSO1* function. First, the TCR motif is highly conserved in two classes of genes (Fig. 4). Second, mutations that substitute a tyrosine for a conserved cysteine in the TCR motif are known to cause severe mutant phenotypes. The strong *tso1-1* and *tso1-2* alleles both result from such a substitution in the conserved cysteine residues (Fig. 4). The temperature-sensitive *Drosophila E(z)*^{61} mutation is also caused by a substitution of a tyrosine residue for a cysteine residue at amino acid 603 of the cys-rich domain (Fig. 4C; Carrington and Jones, 1996). The E(z) protein is part of the polycomb-group complex which interacts with DNA sequences (polycomb response element, PRE) that are scattered throughout the genome (reviewed by Pirrotta, 1998). These interactions can silence the expression of genes containing the PRE as well as nearby genes. At the restrictive temperature, virtually all chromosome binding by this mutant E(z)^{61} protein is eliminated (Carrington and Jones, 1996). Thus, the TCR motif may be required for the interaction with DNA. Third, the two TCR repeats and the sequence connecting them are highly conserved between TSO1 and the soybean CPP1, a negative regulator of the *LEGHEMOGLOBIN* gene expression (E. Ø. Jensen, personal communication). LEGHEMOGLOBINS are nodule-specific proteins involved in buffering the oxygen concentrations in nodules for nitrogen fixation (Bojsen et al., 1983). A single TCR motif from CPP1 is sufficient for low-affinity DNA binding in vitro, but both TCR repeats are required for high-level binding (E. Ø. Jensen, personal communication). The high level of similarity between TSO1 and CPP1 in the TCR repeats and in the

intervening region suggests that TSO1 could bind DNA via its TCR repeats.

We found that *tso1-3* causes early termination of the TSO1 protein, deleting one of the two TCR repeats. As the *tso1-3* phenotype is significantly weaker than that of *tso1-1* and *tso1-2*, it is unclear why removal of one of the two TCR repeats is less detrimental than a single cysteine substitution. The recessive nature of both *tso1-1* and *tso1-2* alleles argue against the possibility that the two strong alleles are dominant-negative or gain-of-function alleles. The intermediate phenotype of *tso1-1/tso1-3* heteroallelic mutants (Hauser et al., 1998) also suggests that both the strong *tso1-1* and the weak *tso1-3* mutants lose *TSO1* activity to different degrees. Weak mutant phenotypes caused by more severe molecular lesions have been previously found in *clv1-6* (Clark et al., 1997) and *sup-5* mutants (Gaiser et al., 1995; Sakai et al., 1995). In this case, a cysteine to tyrosine substitution could perhaps affect the overall conformation of the TSO1 protein, or interfere with the function of both TCR repeats via intramolecular interactions, while a truncated TSO1-3 protein could still function partially via its one TCR motif.

Tissue-specific defects of *tso1* mutants

Previous studies indicated that the defects exhibited by strong *tso1* mutants were largely restricted to flowers and inflorescence meristems (Liu et al., 1997). Examination of roots, vegetative meristems, and leaves failed to reveal any obvious defects. Several hypotheses may explain this largely tissue-specific effect of *tso1* mutations. These hypotheses are not mutually exclusive. First, different tissues or organs may have different requirements for the *TSO1* activity and thus may exhibit different degrees of sensitivity to the loss or reduction of *TSO1*. The ovules may be the most sensitive organs, and the floral tissues may be more sensitive than the vegetative tissues. This hypothesis is consistent with our detection of a higher *TSO1* mRNA level in ovules (Fig. 6E). Second, based on the characterized molecular lesions, none of the *tso1* alleles we have isolated appears to be a complete loss-of-function (null) allele. Thus, defects in other tissue types or embryonic lethality may not be revealed. Third, the function of *TSO1* may be redundant in other non-floral tissues (for example, roots and young seedlings), and the redundant factor is more or is solely active in these non-floral tissues. The *Arabidopsis TSO1-like* gene could be a candidate gene coding for the redundant factor due to its high level of sequence similarity to *TSO1*. Finally, a tissue-specific post-translational regulatory mechanism or alternative transcriptional regulation may underlie the tissue-specific effect of *tso1*.

In our current study, two *TSO1* transcripts were detected that apparently result from differential transcriptional initiation. However, these two transcripts are regulated differently in different tissues. Both transcripts are weakly expressed in roots, seedlings, and flowers (tissues with high levels of cell division activity). However, only the short transcript is expressed in leaves and stems. Such differential transcriptional regulation may be mediated by specific trans-acting factors present in different tissues. Although protein products from both of these TSO1 transcripts are targeted to the nucleus (Fig. 7), it remains to be seen if the two transcripts are functionally distinct and if this differential expression contributes to the largely floral and inflorescence-specific defects of strong *tso1* mutants.

The role of *TSO1* in floral cell division

Our previous analyses showed that *tso1-1* floral meristem cells had incomplete cell walls, increased DNA contents, and multinucleated cells. Based on these defects, it was originally speculated that *TSO1* was directly involved in mitosis, cytokinesis and/or in the coordination between mitosis and cytokinesis. While genes with similar subcellular defects (such as *GNOM/EMB30* and *KN)* encode components of cell secretion machinery (Shevell et al., 1994; Lukowitz et al., 1996), our molecular isolation and analyses of the *TSO1* gene suggest that *TSO1* may encode a nuclear protein that may interact with DNA or chromatin and may play a regulatory rather than a structural role. Furthermore, the observation that *TSO1* mRNA is present in actively dividing cells in developing floral organs supports a role of *TSO1* in promoting cell division, probably by regulating the transcription of genes needed for cell division. The more highly expressed level of *TSO1* mRNA in the primordia of petals, stamen and carpels in a flower is consistent with the phenotype of strong *tso1* mutant flowers, where these organs fail to develop (Liu et al., 1997).

In higher plants, meristem organization and cell division regulation are two fundamentally important and intimately related biological processes. *tso1* is unique in that it affects both of these processes. The effect of *tso1* mutations on cell division in floral meristem appears drastically different from the effect of *tso1* mutations on meristem fasciation. For one, the number of cells formed in each floral meristem is decreased in *tso1-1* (Liu et al., 1997), while the inflorescence meristem fasciation in strong *tso1* alleles causes an increased number of inflorescence meristems and thus an increase in the total number of cells. For another, the defect in the floral meristem appears to reside in the basic cell division processes while the defect in the inflorescence meristem resides in the regulation of meristem organization and partition. These seemingly different defects may be due to differences in the two types of meristems. The floral meristem is a determinate meristem that is fated for floral organ differentiation while the inflorescence meristem is an indeterminate meristem programmed for self-regeneration. Hence, *TSO1* may regulate several downstream genes some of which may function in floral meristems while others may function in inflorescence meristems. Alternatively, these two meristems may manifest the same basic molecular defect differently in different developmental context.

The role of *TSO1* in inflorescence meristem development

When we further characterized the defects of *tso1* mutants in inflorescence meristem fasciation, we found that, instead of a simple enlargement of the inflorescence meristem, fasciation in *tso1* mutants results from repeated division of one inflorescence meristem into two or more inflorescence meristems. This is in contrast to *clv* mutants whose fasciation results from a single large shoot apical meristem (SAM) with more undifferentiated cells (Clark, et al., 1993, 1995; Kayes and Clark, 1998). The fasciated SAM in *clv* mutants grows into a massive line with floral meristems developing along the sides of the apical meristem line. Hence, although both *clv* and strong *tso1* mutants exhibit fasciated inflorescence meristem, their different characteristics suggest that *TSO1* and *CLV* may regulate different aspects of inflorescence meristem development. *CLV* may regulate the size of the central zone by

regulating the rate of cell proliferation and cell differentiation. In contrast, *TSO1* may regulate the number of central zones in each inflorescence. *TSO1* may normally act to limit central zone initiation either by repressing the expression of genes that normally activate central zone formation or by mediating communications between the central zone and its neighbors to prevent additional central zone initiation. For example, in wild-type, the absence or reduction of *TSO1* transcripts in the L1 and L2 layers of the central zone (Fig. 6A) may allow such a central zone to form; while the presence of *TSO1* transcripts in the peripheral zone prevents the initiation of additional central zones. In strong *tso1* alleles, the loss or reduction of *TSO1* activity in the entire inflorescence meristem may allow spontaneous activation of additional central zones. Each new central zone may act as an organizing center and re-establish a peripheral zone that surrounds it.

In summary, the molecular isolation and expression of *TSO1* together with analyses of *tso1* mutant phenotype permit us to establish models on how *TSO1* may function to regulate *Arabidopsis* reproductive development. *TSO1* encodes a putative transcriptional regulator that activates or represses the expression of genes with functions in cell division and inflorescence meristem organization. In wild-type, the absence or reduction of *TSO1* expression in the L1 and L2 layers of a central zone may allow the formation of a single central zone and hence a single inflorescence meristem. In strong *tso1* loss-of-function mutants, the absence of *TSO1* activity in the entire inflorescence meristem may allow the initiation of multiple central zones and thus multiple inflorescence meristems. Our molecular genetic studies have thus established *TSO1* as a critical regulatory protein for meristem development, and indicated that *TSO1* regulates a different and important aspect of inflorescence meristem development distinct from the *CLV* class of meristem regulators.

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