The Plant Cell, Vol. 5, 297–309, March 1993 © 1993 American Society of Plant Physiologists

The Patterns of Gene Expression in the Tomato Shoot Apical Meristem

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In this paper, we describe the synthesis of a cDNA library from the vegetative shoot apical meristem and the analysis of clones selected from it. Using in situ hybridization, we characterized the patterns of expression of these genes in the tomato shoot apical meristem, as well as the patterns obtained from other sources. The results from the analysis of 15 cDNAs indicated the following six main patterns of gene expression in the shoot apical meristem: overall expression, zero expression, expression limited to the epidermis, expression excluded from the epidermis, punctate expression, and expression elevated in the flanks of the meristem. The patterns observed and the nature and number of the genes showing these patterns necessitate a reinterpretation of the models of meristem structure and function. In particular, the data suggest a compartmentation within the shoot apical meristem based on the spatial expression of particular subsets of genes. This paper also reports on the specific and precise criteria essential for the correct identification of meristem-specific gene expression. The data give new insight into the molecular organization of the shoot apical meristem and provide the framework for a detailed dissection of the factors controlling this organization.

INTRODUCTION

The shoot apical meristem plays a vital role in plant development (for reviews, see Steeves and Sussex, 1989; Lyndon, 1990; Medford, 1992). Not only is it the ultimate source of all of the cells of the aerial part of the plant, but the cell divisions that occur in this area are organized to define the initial steps in leaf and stem morphogenesis. In addition, the apical meristem seems to be both the source of signals determining developmental processes spatially removed from the meristem (e.g., apical dominance) and the site of perception of signals that determine the fate of the apical meristem itself (e.g., flowering). These essential functions of the apical meristem have drawn the interest of developmental biologists over many years. The precise definition of a meristem has evolved during this time, and in this paper we have adopted the concept that the shoot apical meristem is that portion of tissue distal and centric to the last visibly formed leaf primordium (Steeves and Sussex, 1989; Medford, 1992). This definition thus distinguishes between a cell merely being meristematic (i.e., capable of undergoing cell division) and a cell being a component of a meristem (in which cell division is an essential but not exclusive requirement). Concomitant with this development in meristem nomenclature, there has been a progressive complexity in the number of different models and interpretations of meristem structure and function (Schmidt, 1924; Satina et al., 1940; Plantefol, 1947; Popham, 1951; Gifford and Corson, 1971). These are summarized in Figure 1.

Early observations on the apical meristem led to the concept that it was organized into a central core of cells, the corpus, overlaid with a mantle of anticlinally dividing cells, the tunica. This model, which is shown in Figure 1B, later became incorporated into one in which the apical meristem can be understood as a series of cell layers derived from a number of initial cells located toward the tip of the apical meristem dome. These layers of the meristem (normally defined as LI, LII, and LIII) are the progenitors of the mature tissues of the plant. Another group of models describing the structure and function of the apical meristem portrays the organ as one divided into a number of distinct zones, independent of any clonal cellular relationships. These models, generally based on histological interpretations, define a peripheral zone on the flanks of the apical dome surrounding a central zone, both of which rest upon a lower rib zone where a transition from meristem to nonmeristem tissue occurs, as is shown in Figure 1C. The peripheral zone is perceived as defining the zone of organogenesis in the apical meristem (i.e., the region where leaf primordia arise), whereas the central zone is thought to act as a source of new cells to replace those gradually lost from the peripheral zone during its organogenic function. The interpretation of the cytological and histological data supporting these different models has often been open to dispute, and, moreover, the significance of the observed patterns with respect to the function(s) of the apical meristem has often been keenly debated (Steeves and Sussex, 1989, p. 88).

Recently, a number of investigators have reported on attempts to identify genes exclusively expressed within the apical

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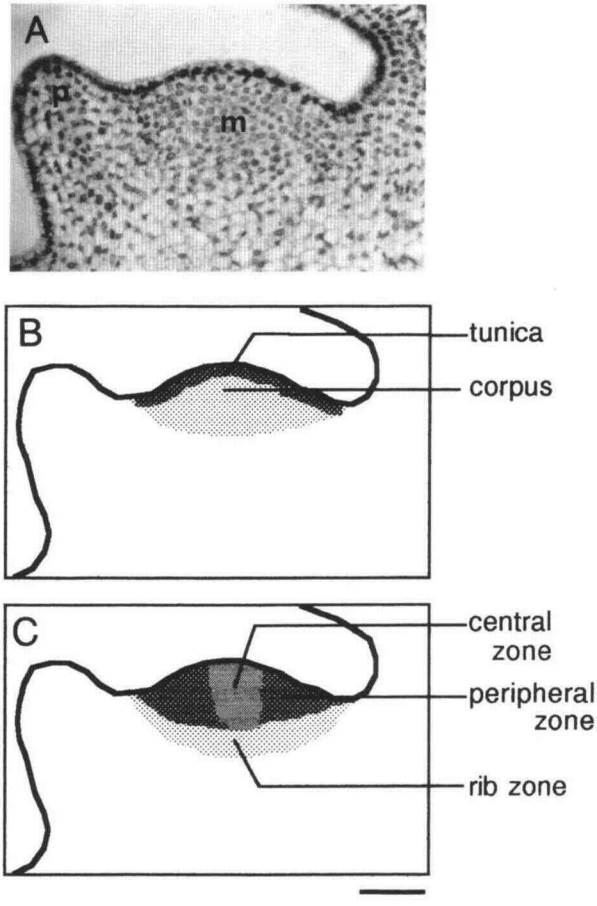

Figure 1. Models of the Shoot Apical Meristem.

(A) Bright-field micrograph of a longitudinal section through a tomato shoot apical meristem. The section has been stained with toluidine blue. m, apical meristem; p, leaf primordium.

(B) Tunica/corpus model. A diagrammatic representation of the micrograph shown in **(A)** with the areas of the tunica and corpus layers indicated.

(C) Zonal model. A diagrammatic representation of the micrograph shown in **(A)** with the areas of the peripheral, central, and rib zones indicated. Bar = 50 μm.

meristem (Melzer et al., 1990; Medford et al., 1991; Kohler et al., 1992; Pri-Hadash et al., 1992). A common experimental approach has been to synthesize cDNAs from apex-derived RNA and then to identify apical meristem-specific genes via screening of subtracted libraries. So far, these approaches have failed to identify any truly meristem-specific clones, and indeed most of the cDNAs identified have encoded proteins with a housekeeping function, e.g., ribosomal proteins and histones. One possible reason for the difficulties encountered in these studies is the fact that the apical meristem proper has often contributed only a small percentage of the tissue used to isolate meristem-specific genes. For example, the contribution of the true apical meristem to the total amount of tissue in a dissected apex consisting of the first three or four primordia is probably less than 1%. In such analyses, the vast excess of non-meristem–derived transcripts is likely to mask and hinder the identification of any apical meristem-specific clones. Furthermore, a lack of clarity on the precise definition of the expected expression pattern of an apical meristem-specific gene has led to some confusion (Medford, 1992). It is evident to us that the identification of specific patterns must by its very nature involve the description or assumption of a defined nonspecific pattern.

In this paper, we describe the construction of a cDNA library from RNA extracted from dissected apices of tomatoes in which the apical meristem tissue was enriched to a level of ~70 to 90% of the total sample. We then used this library to identify genes expressed in particular regions of the apical meristem. Our aims were to define which patterns of gene expression exist within the shoot apical meristem and to use these data to describe the parameters that would define a gene as being truly meristem specific. Our data indicate that the zonation and layered patterns previously described by histological and clonal analysis reflect a spatial regulation of gene expression within the shoot apical meristem. They also delineate the strict criteria that must be met before a gene can be identified as meristem specific.

RESULTS

Dissection of the Apical Meristem

The apical meristem is a dynamic structure whose size and shape change both during a plastochron (the time interval between the production of successive leaf primordia) and during the aging of a plant as it approaches flowering. To allow for a future temporal analysis of gene expression during meristem maturation, we decided to dissect apical meristem tissue from plants undergoing the sixth plastochron. Under our growth conditions, the tomato plants only started to produce flower buds after the 11th to 13th leaf primordium had been produced; thus, the sixth plastochron represented a mid-vegetative stage, well before any overt transition of the meristem toward a floral stage of development (Hussey, 1963). The dissection of the apical meristems from plants of the requisite developmental stage was facilitated by the construction beforehand of a plastochron index, as shown in Figure 2A (Erickson and Michelini, 1957). This defined the sixth plastochron as that stage when, under our growth conditions, the second leaf had an axis length of 28 to 45 mm and the third leaf a length of 5 to 14 mm. Thus, the simple and rapid measurement of axis length for leaf numbers 2 and 3 allowed an easy assessment of whether an individual plant was worth the effort of meristem dissection. This appraisal enabled efficient direction of time toward this labor-intensive stage of the project.

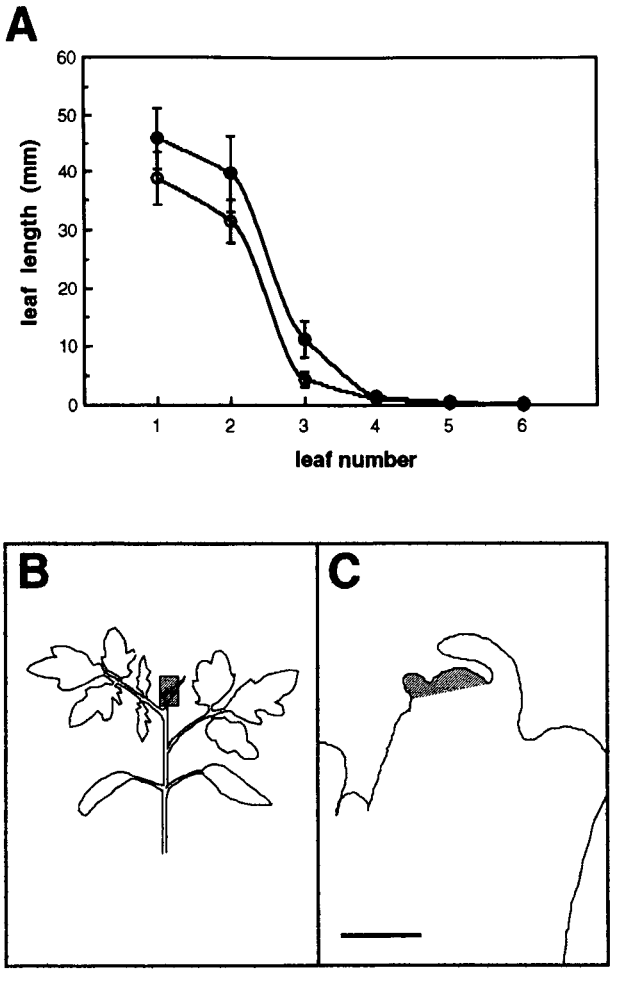

Figure 2. Tissue Dissection for the Meristem cDNA Library.

(A) Plastochron index of tomato plants during growth of the sixth leaf. Plants were dissected and the length of each leaf recorded. (○), plants at an early stage of the sixth plastochron (sixth primordial length 0 to 100 μm); (●), plants at a late stage of the sixth plastochron (sixth primordial length 150 to 250 μm). Error bars are shown when larger than the symbol. The two lines represent the boundary limits in terms of leaf lengths for plants undergoing the sixth plastochron. For construction of the cDNA library, only plants with a second leaf length of 28 to 45 mm and a third leaf length of 5 to 14 mm were selected for meristem dissection.

(B) and **(C)** Meristem dissection. **(B)** Shows a diagram of a young tomato plant with the apical region boxed. This region was cut from plants and then dissected further to reveal the apical meristem. A diagram of a longitudinal section through such a dissected apex is shown in **(C)**. The apical meristem and the youngest primordium arising from it (area shaded) were then removed for the extraction of RNA. Bar = 200 μm.

The tissue collected for RNA extraction is shown diagrammatically in Figures 2B and 2C. It included both the apical meristem and the youngest primordium that was developing on the flank of the meristem. We did not attempt to remove this primordium, but we estimate that the true apical meristem represented between 70 and 90% of the tissue that we collected for the basis of the cDNA library.

Construction of a Meristem cDNA Library and Insert Isolation

From ~300 apical meristems dissected, we extracted total RNA, enriched this sample for poly(A^+) RNA, and then used the estimated 50 ng RNA remaining for the synthesis of a cDNA library. The library was cloned into λZAPII vector, titered, and then subjected to one round of amplification. The titer of the initial library indicated ~40,000 pfus, of which 90% contained inserts.

Our initial question was whether these inserts represented independent mRNAs present in the original tissue. To test for this, we selected a number of clones at random from portions of the amplified library that had been in vivo excised and sequenced the 5′ and 3′ ends of the corresponding inserts. Of the 10 clones subjected to partial DNA sequence analysis, four showed significant similarity to sequences already present in the data bank. These sequences all encode mRNAs whose corresponding proteins might be expected to be present at high levels in metabolically active tissue, such as an apical meristem.

Sequence Analysis of cDNAs Isolated from the Meristem Library

The sequences of three full-length clones isolated from the apical meristem cDNA library are shown in Figure 3. Two of the clones (*T*omato *m*eristem, *Tm1* and *Tm2*) show a high sequence similarity to ribosomal proteins, the other, *Tm3*, has the characteristics of a *ras*-related small GTP binding protein. A fourth partial clone that we have isolated from the meristem library, *Tm4*, shows significant sequence similarity to arginine decarboxylase. The full analysis of this clone will be presented elsewhere. A partial sequence of *Tm4* shows 52% amino acid identity to barley arginine decarboxyase over a stretch of 138 amino acids (amino acids 150 to 288) of the published sequence (Bell and Malmberg, 1990).

The *Tm1* cDNA (Figure 3A) shows a very high sequence similarity to protein L2 of the cytoplasmic ribosomal 60S subunit isolated from barley (Kohler et al., 1992). The 800-bp cDNA insert contains an opening reading frame of 261 amino acids, whose theoretical translation product shows 92% identity and 97% similarity to the barley ribosomal protein L2. We have thus designated *Tm1* as tomato *rpl2*. The mRNA hybridized to by this cDNA in RNA gel blots is ~900 nucleotides long. Clone

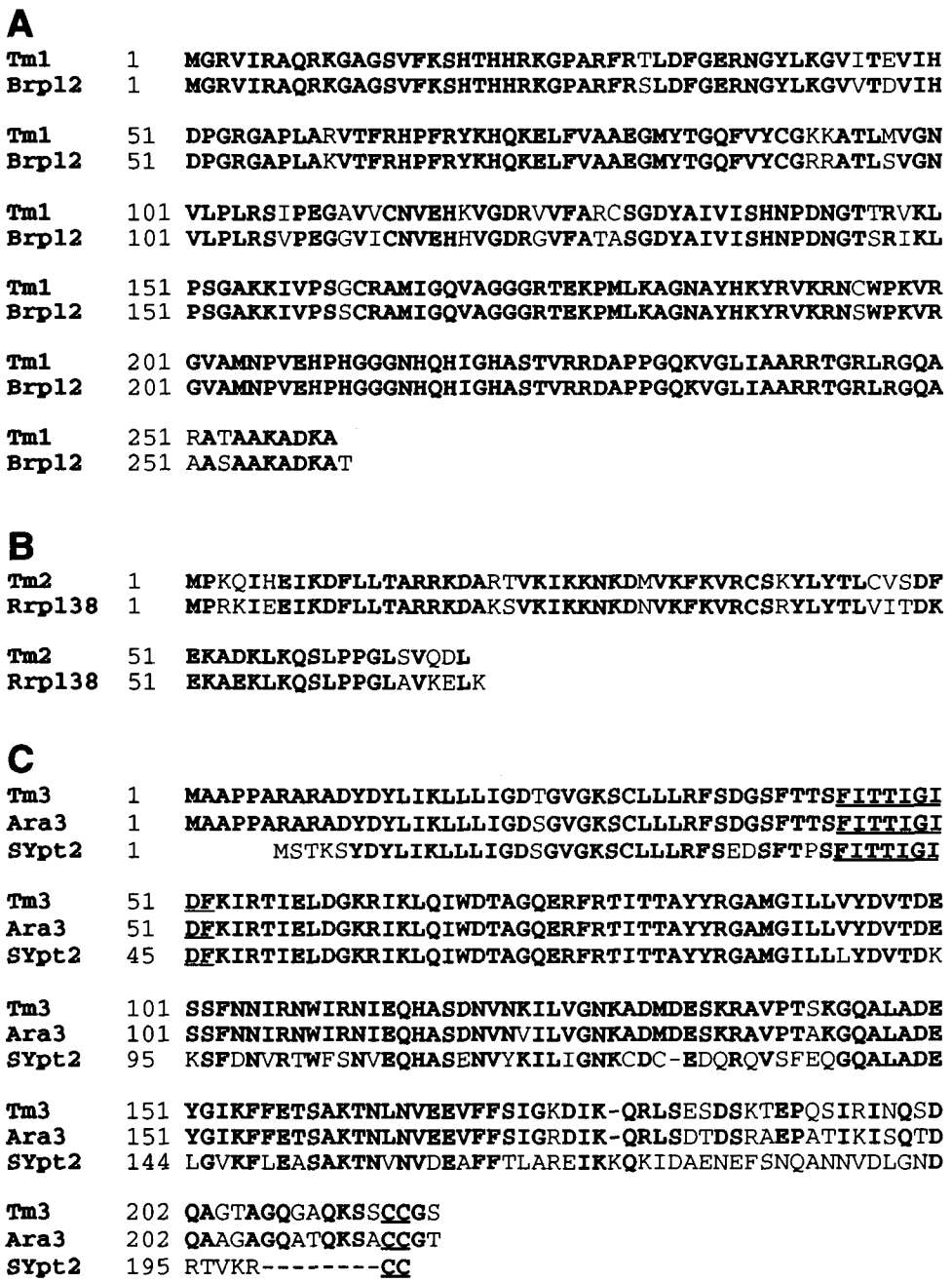

Figure 3. Amino Acid Sequence Similarity of Clones Obtained from the Meristem cDNA Library with Those Already Published.

(A) Comparison of the *Tm1* cDNA with barley ribosomal protein L2, *Brpl2*.
(B) Comparison of the *Tm2* cDNA with the rat ribosomal protein L38, *Rrpl38*.
(C) Comparison of the *Tm3* cDNA with the *Arabidopsis ras* protein, *Ara3*, and the *S. pombe Ypt2* protein.
In each case, the deduced amino acid sequence of the Tm clone is shown above the amino acid sequence of the protein with which it is being compared. Residues identical in the Tm clones with those of other proteins are in bold print. In **(C)**, the putative ras "effector region" at amino acid positions 44 to 52 (*Tm3* and *Ara3*) and 38 to 46 (*SYpt2*), and the two cysteine residues toward the C-terminal end of the proteins thought to be involved in palmitoylation/isoprenylation have been underlined. The nucleotide sequences reported above have been submitted to the EMBL data base as accession numbers X64562 for tomato *rpl2*, X69979 for tomato *rpl38*, and X69980 for tomato *ypt2*.

Table 1. Identity of Tm Clones

Clone	Homology with:	% Protein Identity
Tm1	Barley ribosomal protein L2 (Kohler et al., 1992)	92
Tm2	Rat ribosomal protein L38 (Kuwano et al., 1991)	82
Tm3	Arabidopsis GTP binding protein, *ara3* (Anai et al., 1991)	94
	S. pombe GTP binding protein, *ypt2* (Haubruck et al., 1990)	74

Tm2 (Figure 3B) also encodes a protein component of the cytoplasmic 60S ribosomal subunit, in this case showing an identity of 82% at the amino acid level with the rpl38 protein isolated from rat (Kuwano et al., 1991). This cDNA, which we have designated tomato *rpl38*, is 450 bp long and corresponds to a transcript of 500 nucleotides observed in RNA gel blots. The third clone, *Tm3*, whose sequence is shown in Figure 3C, demonstrates sequence similarity with a G protein, *ara3*, isolated from Arabidopsis (Anai et al., 1991). This similarity (94% at the amino acid level) suggests that *Tm3* encodes for a member of the *ras* gene subfamily termed *ypt*. This similarity extends to the effector region (involved in GTP binding) around amino acid 45, which contains the sequence Phe-Ile-Thr-Thr-Ile-Gly-Ile-Asn-Phe observed in both the *Schizosaccharomyces pombe ypt2* (Haubruck et al., 1990) and the *Arabidopsis thaliana ara3* genes. The *Tm3* cDNA also contains two cysteine residues toward the C terminus of the predicted protein. This motif is characteristic of small GTP binding proteins and is thought to be the site of palmitoylation/isoprenylation (Balch, 1990). The *Tm3* cDNA shares 94% identity with the Arabidopsis *ara3* gene and 74% identity with the *S. pombe ypt2* gene. We have therefore designated this clone as tomato *ypt2*. In yeast, ypt2 protein has been shown to be localized to the Golgi apparatus, where it is thought to carry out an essential function in vesicle transport. The similarities of the *Tm* clones with sequences already published are summarized in Table 1.

Genes Isolated from the Meristem cDNA Library (and Other Sources) Are Differentially Expressed within the Apical Meristem

To identify the patterns of gene expression in the shoot apical meristem, we performed a series of in situ hybridizations using randomly isolated cDNAs from the meristem library. These clones gave rise to a variety of expression patterns. We also extended our study to include a number of genes of known function (kindly sent to us by several colleagues), which we anticipated would be expressed in the apical meristem. The results of this examination of the expression patterns of various genes are shown and summarized in Figure 4 and Table 2.

Figure 4 shows the six patterns of gene expression that we have observed by in situ hybridization in the shoot apical meristem of tomato. Each row in this figure shows a specific pattern as portrayed by a representative cDNA. The picture in the first column shows the structure of the apical meristem, the second column shows the signal observed in the section, and the third column shows a diagrammatic representation of the signal pattern.

Genes belonging to the pattern I group (Figures 4A to 4C), represented here with the cDNA for *rpl2* (*Tm1*), gave a uniform signal over all cells within the meristem. No restriction or preferential expression within the apical meristem was observed. As can be seen from Table 2, this group comprised the majority of the cDNAs that we have studied. These clones represent genes whose products are involved in disparate functions within the cell, including translation, aromatic amino acid synthesis, and transcription initiation (MADS box).

A second pattern (Figures 4D to 4F; pattern II) was obtained using a fragment of the tobacco *Ltp1* gene, which codes for a lipid transfer protein (LTP) (Fleming et al., 1992). This probe gave a high signal only in the outermost cell layer of the meristem. This layer, whose derivatives give rise to the epidermis of the plant, is the LI layer, as defined in the model by Satina et al. (1940). A similar pattern was observed in the tomato apical meristem by Shahar et al. (1992) using a probe homologous to polyphenoloxidase.

The complementary pattern to that displayed by the *LTP* gene is shown in Figures 4G to 4I; pattern III). Here, a high signal can be seen in the innermost cells of the apical meristem, with the signal intensity decreasing toward the outermost cell layers. This pattern is represented by an in situ hybridization using a probe for *Tm4*, which has homology to oat arginine decarboxylase (Bell and Malmberg, 1990). A similar pattern has been reported for a dUTPase cDNA (Pri-Hadash et al., 1992).

Two of the cDNAs isolated from the meristem cDNA library gave rise to an expression pattern in which the flanks of the apical meristem had a very high signal relative to that detected in the central part of the tissue (Figures 4J to 4L; pattern IV). This area of low signal did not extend to the surface of the apical meristem, where a region of high signal intensity can be seen in the uppermost layers of cells covering the tip of the apical dome. This pattern is represented using a probe against the *rpl38* cDNA (*Tm2*). The other cDNA found to show this pattern of gene expression encodes a *ypt2*-like G protein (*Tm3*).

A fifth pattern of gene expression is demonstrated in Figures 4M to 4O (pattern V) with an in situ hybridization using a probe against a histone *H2A* gene. In this pattern, a number of spots of high signal intensity can be seen scattered across the apical meristem. This pattern, listed in Table 2, confirms the observations of Koning et al. (1991), who have reported a similar pattern of histone gene expression in the shoot apex of tomato.

A final pattern that can be distinguished is shown in Figures 4P to 4R (pattern VI). This pattern actually represents the converse of that seen in Figures 4B and 4C (pattern I), that is in

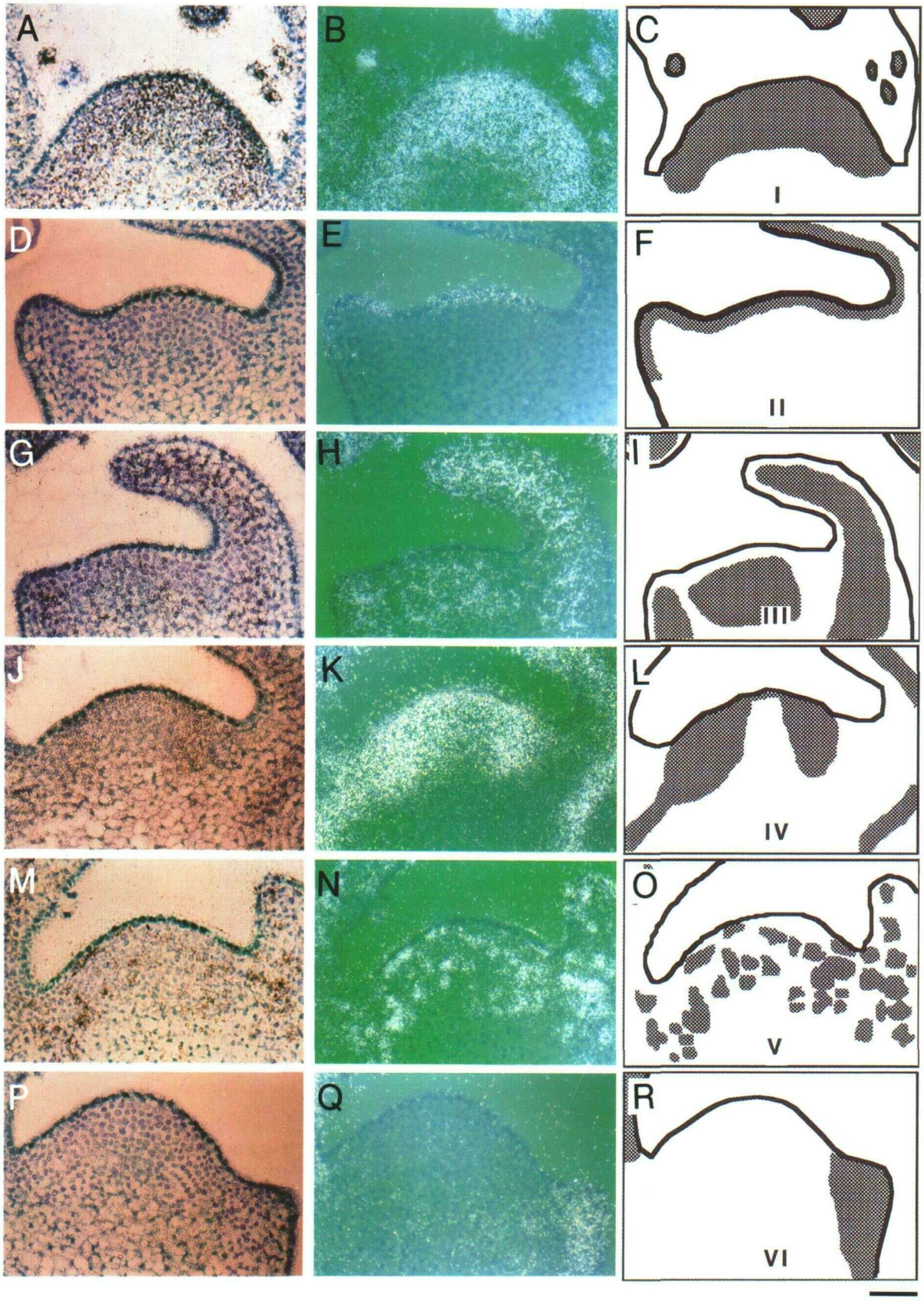

Figure 4. Patterns of Gene Expression in the Apical Meristem.

this case there is no detectable gene expression within the apical meristem. This pattern is demonstrated using a probe for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase.

Definition of the Apical Meristem by Gene Expression Patterns

The specific patterns of gene expression seen in the apical meristem by in situ hybridization are not reflected by any special restriction of the corresponding transcripts to particular organs in the plant, as shown in Figure 5 by RNA gel blots. The six blots in Figure 5 correspond to the six cDNAs used for the in situ hybridizations shown in Figure 4 to demonstrate the patterns of gene expression within the apical meristem. Both the histone and the *Ltp* transcripts seem to indicate some localization to the apex, although analysis of the apices shown in Figures 4E and 4N demonstrate that these transcripts are certainly not specific to the meristem. The two ribosomal protein genes, *rpl2* and *rpl38*, are expressed in all four organs examined in the RNA gel blots, whereas the ribulose-1,5-bisphosphate carboxylase (*rbcS*) transcript is restricted to aerial parts of the plant. The *Tm4* transcript is detectable in all organs but shows an elevated level in the shoot apex and root tissue.

None of the genes obtained from the meristem cDNA library showed exclusive localization to the shoot apical meristem, only particular patterns of expression within it. However, in situ hybridization studies on the whole shoot apex illustrated how, due to the peculiar cytology and histology of this tissue, even genes that are not specifically expressed in the apical meristem can produce patterns which seemingly demonstrate specific localization to the apical meristem. Examples of such hybridizations are shown in Figure 6.

When the shoot apex was hybridized with a probe for *rpl2* (Figure 6A), a very high signal was seen in the apical meristem relative to the subtending tissue, creating the impression of localization to the apical meristem. However, comparison of this pattern to those obtained either by in situ hybridization using an rRNA probe (Figure 6B) or by simply staining the apex with acridine orange (a histological stain for nucleic acids) (Figure 6C) demonstrates that the pattern seen with the *rpl2* probe simply reflects the overall distribution of RNA within the shoot apex. In situ hybridizations with a probe against the *rbcS* gene, however, gave no detectable signal within the apical meristem, although the tissue immediately adjacent to the

Table 2. Patterns of Gene Expression in the Shoot Apical Meristem

Pattern	Characteristics of Expression Pattern	Genes Showing Expression Pattern
I	Expression in all cells of the meristem	Ribosomal protein L2[a] (*rpl2*) Vegetative MADS box gene[b] Basic glucanase[b] Shikimate kinase[b] EPSP synthase[b] 18S rRNA[b] *Tm5*[a] *Tm6*[a] *S1*[b]
II	Expression elevated in the tunica	Lipid transfer protein[b] (LTP) Polyphenoloxidase[1]
III	Expression elevated in the corpus	Arginine decarboxylase[a] (ADC) dUTPase[2]
IV	Expression elevated in the peripheral zone	Ribosomal protein L38[a] (*rpl38*) *ypt2*-like GTP binding protein[a]
V	Expression elevated in cell clusters	Histone 2A[3] Histone 4[b]
VI	No expression within the meristem	Small subunit of Rubisco[b]

The in situ hybridizations were performed either with clones selected from the apical meristem cDNA library (a), or with clones from various other sources (b). The following table entries have been reported in the literature: 1, Shahar et al. (1992); 2, Pri-Hadash et al. (1992); and 3, Koning et al. (1991).

Figure 4. (continued).

Each row in this figure consists of a set of three pictures. The first picture in each row shows a light micrograph of an apical meristem that has been hybridized with an antisense RNA probe against a particular mRNA. The second picture in each row shows the localization of the signal seen with each probe, as visualized by a combination of polarized epifluorescence and bright-field microscopy. The third picture in each row is a diagrammatic representation of the meristem to display the localization of the signal seem by in situ hybridization, as indicated by the region of shading. Bar = 50 μm.

(A), **(B)**, and **(C)** In situ hybridization with the *Tm1* (*rpl2*) probe.
(D), **(E)** and **(F)** In situ hybridization with the *Ltp1* probe.
(G), **(H)**, and **(I)** In situ hybridization with the *Tm4* (*ADC*) probe.
(J), **(K)**, and **(L)** In situ hybridization with the *Tm2* (*rpl38*) probe.
(M), **(N)**, and **(O)** In situ hybridization with a histone *H2A* probe.
(P), **(Q)**, and **(R)** In situ hybridization with an *rbcS* probe.

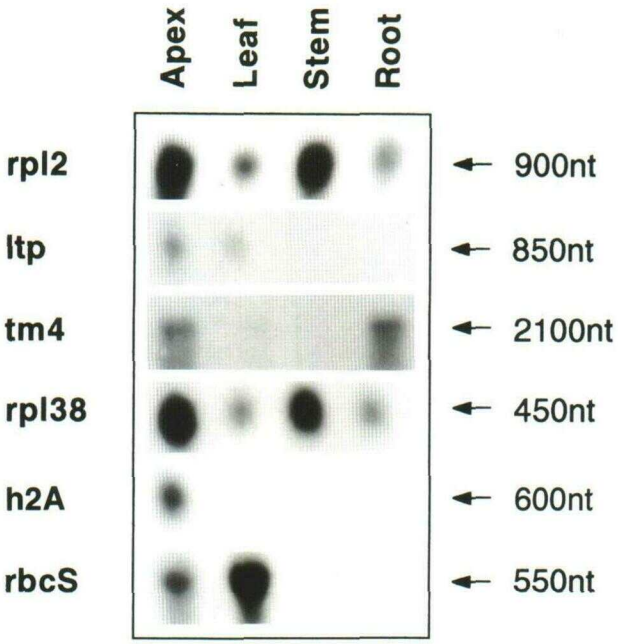

Figure 5. RNA Gel Blot Analysis of mRNAs Showing Specific Patterns of Expression in the Shoot Apical Meristem.

Total RNA was isolated from various parts of young tomato plants and equal amounts (2 μg) glyoxylated and run on agarose gels. Apex, the apical part of the plant with leaves of length less than ~1 cm; Leaf, leaves of length greater than 5 cm; Stem, any internode tissue higher than the first leaf; Root, all nongreen tissue below the hypocotyl. The ^{32}P-labeled probe used for each hybridization is indicated to the left of each row, and the approximate size of the transcript detected is indicated in nucleotides to the right of each row.

apical meristem (i.e., young primordia and subtending shoot tissue) gave rise to a relatively high signal (Figure 6D), with the exception of the vascular tissue.

DISCUSSION

Gene Expression Patterns within the Shoot Apical Meristem

By analyzing the expression of a number of genes, some of which were obtained from an apical meristem cDNA library, we observed a variety of patterns in the apical meristem. The patterns that we observed, when put into the context of the two types of models described in Figure 1, necessitate a reinterpretation both of how the apical meristem is organized at the cellular level and the functional significance of this organization.

The peripheral zonation observed by classic histological techniques has usually been interpreted as reflecting a generally higher level of metabolism in the cells of this region, which are involved in the generation of new organs (Gifford and Corson, 1971; Steeves and Sussex, 1989). However, our analysis indicated that the majority of genes expressed within the apical meristem are expressed equally in all cells, with no apparent localization to the peripheral zone (Table 2). This is

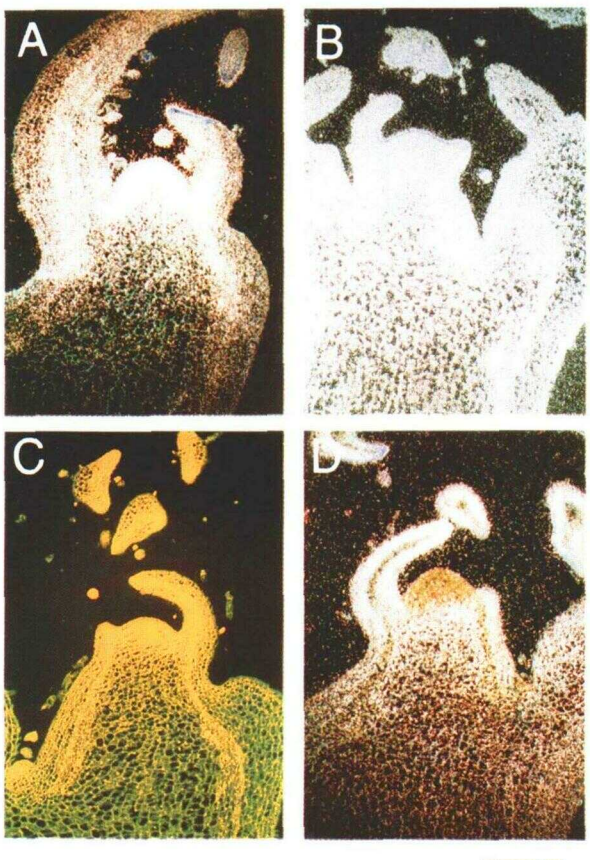

Figure 6. RNA Distribution within the Shoot Apex.

(A) In situ localization of *Tm1* (*rpl2*) transcripts. A tomato apex was fixed and embedded and the *rpl2* mRNA localized in 7-μm sections using a ^{35}S-labeled antisense RNA probe. The signal (silver grains) has been visualized by dark-field microscopy.

(B) In situ localization of mitochondrial rRNA. A tomato apex was fixed and embedded and the rRNA localized in 7-μm sections using a ^{35}S-labeled antisense RNA probe. The signal (silver grains) has been visualized by dark-field microscopy.

(C) Distribution of total RNA. A tomato apex was fixed and embedded and then stained with acridine orange after pretreatment with DNase. The 7-μm section was viewed by epifluorescent microscopy using a barrier filter of 520 nm and an excitation filter of 490 nm. RNA fluoresces orange under these conditions.

(D) In situ localization of *rbcS* transcripts. A tomato apex was fixed and embedded and the *rbcS* mRNA localized in 7-μm sections using a ^{35}S-labeled antisense RNA probe. The signal (silver grains) has been visualized by dark-field microscopy.

Bar = 200 μm.

true for a mitochondrial rRNA (which represents a significant fraction of the total RNA within a cell) and for at least one ribosomal protein (a protein intrinsically linked to translation). Such uniform patterns have been observed repeatedly in several independent experiments using several probes. However, genes with elevated transcript levels within the peripheral zone are present in the apical meristem. For example, in this study we isolated two clones from an apical meristem cDNA library (a ribosomal protein and a G protein) whose mRNAs are present at a higher level on the flanks of the apical meristem.

In the literature, a peripheral zonation pattern has been reported for napin transcripts in the apical meristems of *Brassica napus* embryos (Fernandez et al., 1991) and on the flanks of apical meristems on the transition to flowering in mustard (Melzer et al., 1990). Our data indicated that this observed occurrence of a peripheral zonation pattern at the transcript level is not due simply to the general level of metabolism occurring in these cells; rather, some genes are specifically up-regulated in this region (or down-regulated in the adjacent central zone).

Work on flower mutants in a number of plants has shown that particular regions of the floral meristem may be defined by particular members of a group of transcription factors, termed MADS box genes (Coen and Meyerowitz, 1991). It is tempting to speculate that the observed up regulation of certain genes in the peripheral zone of the vegetative apical meristem is due to the limited spatial expression of some MADS box gene(s). However, our in situ hybridization studies with a vegetatively expressed MADS box gene (*TM3* clone as defined by Abu-Abeid et al., 1991) indicated no localization of the corresponding transcript within a particular region of the apical meristem. We are screening the apical meristem cDNA library to identify other transcription factors expressed in the apical meristem that might specify zonation patterns.

Previous work on the visualization of the different clonal layers that compose the apical meristem has required the construction or identification of chimeras in which one or more of the layers has been genetically marked (Tilney-Bassett, 1986). In contrast, the layered pattern of gene expression that we have recorded (pattern II and pattern III) was observed in nonchimeric plants. This pattern, in which the epidermal layer and the underlying layers are phenotypically but not genotypically distinct, is different from the three-layered structures determined by clonal analysis. It is very similar to the pattern of gene expression reported by Pri-Hadash et al. (1992) and Shahar et al. (1992), and also for the apical meristem of tomato, in which a polyphenol oxidase gene product is localized to the epidermis and a UTPase gene product is excluded from the epidermis.

This phenotypic differentiation of the epidermal layer is, perhaps, not surprising. The epidermis is a specialized structure marking the boundary between the plant and its environment. It is involved in interactions with potentially pathogenic bacteria and fungi, is the first site of damage after insect attack, and is a main site of water loss from the plant. It is also possible that the biophysical characteristics of the epidermal cell wall play a determining or facilitative role in organ morphogenesis (Selker et al., 1992). Specific gene expression might thus be expected in this cell layer (Clark et al., 1992). It is more surprising that certain gene functions seem to be lacking (or at least greatly decreased) from the outermost cell layer of the apical meristem (e.g., ADC, dUTPase). Are these gene functions nonessential for the plant cells in the epidermis (which are dividing and metabolizing), or are the protein products for these genes supplied *in trans* from the underlying cell layers? In neither this study nor that of Pri-Hadash et al. (1992) were gene-specific probes used; therefore, it seems unlikely that there are epidermis-specific members of gene families whose transcripts went undetected in these experiments.

Whereas clonal analysis studies have indicated the presence of three layers within the apical meristem, our data and those of Lifschitz's group (Pri-Hadash et al., 1992; Shahar et al., 1992) indicate a separation of the meristem into two cellular compartments, the epidermis and the cells underlying it. This pattern most resembles the compartmentation proposed from very early studies on the apical meristem, which identified an outer tunica layer enveloping an inner corpus of cells (Figure 1B) (Schmidt, 1924). The significance of this observed separation is unclear to us, other than that it stresses the unique biochemical, and, thus, possibly functional nature of the epidermis.

In this analysis of gene expression in the shoot apical meristem, we identified six major patterns. We cannot discount the possibility that other patterns exist, but we can state that the frequency of the mRNAs showing such patterns must be relatively rare. In particular, we have not characterized any gene whose expression is limited to the central zone of the apical meristem, and we are conducting experiments to identify such cDNAs, if they exist.

Functional Significance of Transcript Localization within the Apical Meristem

Ribosomes contain some 60 proteins that are required in equimolar concentrations for correct ribosome structure and function (Mager, 1988). Thus, at first, it seems surprising that two ribosomal proteins (*rpl2* and *rpl38*, compare Figures 4B and 4K) should show two distinct patterns of gene expression within the apical meristem. However, the data accumulated from investigations on nonplant systems (in particular studies on Xenopus embryo development) suggest that post-transcriptional regulation plays an important role in controlling the expressed levels of different ribosomal proteins (Wormington, 1988). The observed differences in the transcript level of different ribosomal proteins within the apical meristem suggest that similar post-transcriptional mechanisms function in plants, especially in the region of organogenesis.

The observed restriction in the expression of *Tm4* (homologous to arginine decarboxylase), as shown in Figure 4H, is intriguing because the polyamine end products of the metabolic pathway entered via this enzyme have frequently been associated with increased levels of cell division (Evans and

Malmberg, 1989). The apical meristem certainly contains a large number of dividing cells; however, the functional significance of the observed decreased transcript level toward the outer cell layer of the meristem is obscure because there is little evidence that it reflects the recorded distribution of cell divisions (Lyndon, 1976). However, a recent report using animal cells may shed some light on this problem. Auvinen et al. (1992) showed that overexpression of ornithine decarboxylase (a key enzyme in animal polyamine biosynthesis) led to uncontrolled cell proliferation, whereas down regulation of the enzyme induced epithelial characteristics. In comparison, arginine decarboxylase mRNA appears to be expressed at a very low level in the future epidermis in the shoot apical meristem, and up-regulated in the inner corpus cells (an area of the meristem characterized by the lack of a regular orientation in the plane of cell division). How far this analogy between the plant and animal systems can be drawn awaits the investigation of the effect of over- and under-expression of arginine decarboxylase in plants.

Our data indicated that the transcript for an LTP is found only in the outermost cell layer of the apical meristem (Figure 4E). LTPs have been shown by a number of workers to be preferentially expressed in the epidermis (Sossountzov et al., 1991; Sterk et al., 1991; Fleming et al., 1992), and the outermost cell layer of the apical meristem is the progenitor of the epidermis. This localization of LTPs suggests that they might play a role in a specific function of the epidermis, for example, possibly in the synthesis of the lipid rich extracellular cuticle covering this cell layer.

A study of a Brassica histone promoter linked to a β-glucuronidase (GUS) reporter gene in transgenic tobacco plants indicated a localization of the GUS product to the peripheral zone (Medford et al., 1991), although the data presented here and by other investigators using in situ localization of histone transcripts suggest a more punctate distribution of histone mRNAs (Figure 4N; Koning et al., 1991). We interpret this punctate pattern as reflecting areas within the apical meristem where cytological analysis has indicated some localized synchrony of cell division (Lyndon, 1990, p. 32), although it is not possible to discount the occurrence of endoduplication. The apparent anomaly between the transcript localization and the GUS histochemical data could be resolved if one assumes that the pockets of cell division in the apical meristem are localized preferentially over time to the peripheral zone. At any one time, there would be an apparent punctate distribution of the histone transcripts (as observed by in situ hybridization studies), but over a period of time, these transcripts would be expressed in cells localized to the peripheral zone (as observed by histone promoter GUS fusions due to the relative stability of the GUS protein).

Definition of Meristem Specificity

Some of the cDNAs that we have analyzed display an expression pattern that, at first sight, seems to indicate some localization of expression to the apical meristem. Thus, as shown in Figure 6A, the transcripts for the *rpl2* gene are located predominantly in the region of the apical meristem. However, analysis of the distribution of total RNA (Figures 6B and 6C) indicates that this localization of transcripts to the apical region is simply a reflection of the peculiar histology of this tissue. Any specification of a gene as meristem specific must take this histology into account. With respect to the expression pattern of any gene deemed to be meristem specific, a number of conditions must be met. First, the transcript must be shown to be localized to the cells of the meristem and not to be expressed in any of the cells derived from the meristem, i.e., no detectable transcript levels in any cells of the stem or youngest primordium arising from the apical meristem. Such precision requires either the use of in situ hybridization techniques or polymerase chain reaction–based analysis of precisely dissected meristem and nonmeristem tissue. Second, control studies using probes known to be nonmeristem specific should be performed to establish that these probes give a signal in nonmeristem tissue by the analytical method used. To our knowledge, so far no gene has been shown to fulfill the criteria described.

Transcripts for the *rbcS* gene are not detectable in the apical meristem (Figure 6D) but are detectable in all cells just proximal to the apical meristem, with the exception of some cells of the vascular tissue. Although the *rbcS* genes are known to be regulated by light (Kuhlemeier et al., 1987; Gilmartin et al., 1990), it has been shown that there is also a developmental component to the regulation of these genes (Sugita and Gruissem, 1987; Kuhlemeier, 1992). The sharp transition between cells expressing or not expressing the *rbcS* transcripts in the shoot apex suggests to us that the *rbcS* genes are under a strict developmental control, and that at least part of this control resides in whether a cell is in the apical meristem or not. It cannot be simply a case of whether a cell is meristematic or not, because many of the cells in the young primordia expressing the *rbcS* genes are still undergoing cell division. By this interpretation of the in situ hybridization shown in Figure 6D, the *rbcS* transcript pattern negatively defines the shoot apical meristem.

In summary, our data provide a framework for the future analysis of gene expression in the shoot apical meristem. They define the patterns that can be expected to be observed for any gene expressed within this region and thus identify the future lines of research on the characterization of how these patterns are specified and what might be the effect of pattern disruption on meristem function.

METHODS

Meristem Dissection and cDNA Library Construction

For the construction of a meristem cDNA library, the apical meristems of tomato plants (*Lycopersicon esculentum* cv Moneymaker) were

dissected and collected in liquid nitrogen. The dissection involved the removal of all leaf primordia and associated stem tissue from each plant apex, except for the youngest primordium arising from the meristem itself (Figure 2C). The tissue collected comprised ~70 to 90% truly apical meristem tissue. The plants used for dissection were staged by use of a plastochron index (Erickson and Michelini, 1957) constructed during trial dissections (Figure 2A). Thus, only plants with a second leaf length of 28 to 45 mm and a third leaf length of 5 to 14 mm were chosen for dissection. Approximately 300 plants were dissected to give a total meristem fresh weight estimated at 2 mg, collected in a 500-μL microcentrifuge tube.

Total RNA was extracted from the meristem tissue sample by the method of Logemann et al. (1987) in a total volume of 50-μL extraction buffer (8 M guanidine-HCl, 20 mM Mes, pH 7, 20 mM EDTA, 2% (v/v) β-mercaptoethanol). After phenol-chloroform extraction and centrifugation for 45 min, the nucleic acids were precipitated with acetic acid and ethanol. The pellet was washed two times with 3 M sodium acetate, pH 5.2, one time with 70% ethanol, and then resuspended in 10 μL water. Estimation of the nucleic acid content of this sample using a DNA Dipstick (Invitrogen, San Diego, CA) indicated a total amount of RNA of ~5 μg. This RNA sample was enriched for poly(A^+) RNA by one passage through oligo-dT 77 (Pharmacia) using a self-made minicolumn. The poly(A^+) RNA–enriched fraction (estimated to contain ~50 ng of nucleic acid) was precipitated using glycogen as a carrier. The pellet was dissolved in 5 μL water, and the entire sample was used in the synthesis of double-stranded cDNA using a kit from Pharmacia. EcoRI/NotI adapters were added to the cDNA, according to the manufacturer's instructions. Then the entire product of the cDNA synthesis was ligated into the vector phage λZAP II. Ligation and packaging (Gigapack Gold) were performed according to the manufacturer's instructions (Stratagene), and the library was plated on *Escherichia coli* K12 XL1-Blue.

cDNA Insert Isolation and Analysis

An aliquot of the library was excised in vivo to produce Bluescript phagemids containing the cDNA inserts, according to the manufacturer's instructions (Stratagene). This pool of excised phagemids was then used to infect bacterial cells and was plated overnight. From the large number of resulting colonies, 24 were picked at random and the cDNA inserts purified. Those inserts with a size greater than 300 bp (12) were then partially sequenced from the 5′ and 3′ of the cDNA ends and a sequence homology search was made. Double-stranded DNA sequencing was performed using the dideoxy chain termination method.

RNA Gel Blot Analysis

Total RNA was extracted with guanidinium thiocyanate from various organs of young tomato plants, followed by centrifugation through a caesium chloride solution (Maniatis et al., 1982). Aliquots of 2 μg were glyoxylated (Hull, 1985), and then run on 1.2% agarose gels before transfer to nylon membranes (Nytran; Schleicher and Schuell). After deglyoxylation and fixation by baking and a UV light treatment, blots were prehybridized (3 hr) and hybridized (14 to 16 hr) in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1 × PE (50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 25 mM EDTA, 0.2% BSA), 50 μg/mL yeast tRNA, and 50% deionized formamide at 65°C. Phosphorus-32–labeled antisense riboprobes were synthesised from the appropriate DNA template for each cDNA analyzed (Stratagene) and added to the hybridization mix. Blots were washed two times at room temperature with 2 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 0.1% SDS, and then once at 65°C with 1 × SSPE, 0.1% SDS for 15 min, before a final wash also at 65°C for 15 min with 0.1 × SSPE, 0.1% SDS. Blots were exposed against x-ray film with intensifying screens at −80°C for between 2 hr and 3 days before development. The amount of RNA and the integrity of the ribosomal RNA were confirmed by methylene blue staining of blots made in parallel to those used in the hybridizations.

In Situ Hybridizations

In situ hybridizations were performed essentially according to the method of Cox and Goldberg (1988). Briefly, tomato apices from young plants (5 to 7 plastochrons old) were fixed with 4% formaldehyde (w/v), 0.25% (w/v) glutaraldehyde in phosphate buffer, pH 7.2; then, after dehydration in ethanol and exchange with xylene, they were embedded in paraffin. Sections (7 μm) were cut and attached to slides coated with poly-L-lysine. The slides were subjected to a prehybridization treatment of proteinase K followed by acetic anhydride, before hybridization with ^{35}S-labeled riboprobes. Hybridizations were conducted overnight at 42°C. The slides were washed four times with 4 × SSC at room temperature, 30 min at 37°C in a 1 μg/mL solution of RNase A, and finally washed at room temperature in 2 × SSC. Slides were then coated with Kodak ND4 x-ray emulsion before exposure at 4°C for 6 days to 3 weeks prior to development. After development, the sides were stained in toluidine blue and then viewed under dark- and bright-field light microscopy and by polarized epifluorescence microscopy using an IGS filter block (Nikon). In all cases, control hybridizations were performed with the corresponding sense probes, and in all instances the signal obtained was negligible compared to that obtained using the antisense probe. Care was taken to view those sections at or near the mid-point of each meristem examined so as to ensure that the patterns observed were due to the specific probe being used and not due to the particular section through the meristem.

Some slides were not subjected to hybridization but were stained with a 0.1% solution of acridine orange for 30 sec, washed in 50-mM calcium chloride, dried, and then viewed by epifluorescence microscopy using an excitation filter of 490 nm and a barrier filter of 520 nm (Nikon). To assess the amount of acridine orange binding to RNA (as opposed to DNA), some slides were treated with RNase A prior to staining.

Source of cDNAs Used for In Situ Hybridizations

The following clones used in this work were generously provided by our colleagues: the cDNAs for shikimate kinase, EPSP synthase, and cDNA S1 (unknown function) were kindly donated by Dr. Jürg Schmid (ETH, Zürich). The rRNA probe was the generous gift of Dr. Mike Saul (also of the ETH). Dr. Regina Vögeli-Lange (FMI, Basel) provided the cDNA for the basic β-glucanase; the *rbcS* clone was sent to us by Dr. Wilhelm Gruissem (University of California, Berkeley), and the H2A cDNA was the gift of Dr. Luca Comai (University of Washington, Seattle). The H4 cDNA was kindly provided by Dr. Klaus Theres (University of Cologne). All other clones used were obtained from the apical meristem cDNA library, with the exception of the MADS box gene which we obtained by a polymerase chain reaction strategy from tomato RNA using primers designed from the sequence published by Abu-Abeid

et al. (1991) for the vegetatively expressed MADS box clone, *TM3*. The *Ltp1* probe used in this study was the same as that described in Fleming et al. (1992).

ACKNOWLEDGMENTS

This work was supported by grants from the Schweizerischer Nationalfonds and Stiftung zur Förderung der wissenschaftlichen Forschung an der Universität Bern to C.K. A.J.F. would also like to thank Dr. Cheryl Smart (ETH, Zurich) for useful discussion, critical reading of the manuscript, and the preparation of the artwork.

Received January 4, 1993; accepted January 25, 1993.

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