

The development of apical embryonic pattern in *Arabidopsis*

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SUMMARY

The apical portion of the *Arabidopsis* globular stage embryo gives rise to the cotyledons and the shoot apical meristem (SAM). The *SHOOT MERISTEMLESS (STM)* gene is required for SAM formation during embryogenesis and for SAM function throughout the lifetime of the plant. To more precisely define the development of molecular pattern in the apical portion of the embryo, and the role of the *STM* gene in the development of this pattern, we have examined *AINTEGUMENTA (ANT)*, *UNUSUAL FLORAL ORGANS (UFO)* and *CLAVATA1 (CLV1)* expression in wild-type and *stm* mutant embryos. The transcripts of these genes mark subdomains within the apical portion of the embryo. Our results indicate that: (1) the molecular organization characteristic of the vegetative SAM is not present in the

globular embryo but instead develops gradually during embryogenesis; (2) radial pattern exists in the apical portion of the embryo prior to and independent of *STM* with *STM* expression itself responding to radial information; (3) the embryonic SAM consists of central and peripheral subdomains that express different combinations of molecular markers and differ in their ultimate fates; and (4) *STM* activity is required for *UFO* expression, *STM* is required for maintenance but not onset of *CLV1* expression and the pattern of *ANT* expression is independent of *STM*.

Key words: Meristem, *Arabidopsis*, Plant development, *SHOOT MERISTEMLESS*, Embryogenesis, *AINTEGUMENTA*, *UNUSUAL FLORAL ORGANS*, *CLAVATA1*, Stem cells

INTRODUCTION

The shoot apical meristem (SAM) of angiosperm plants gives rise to new leaves and stem in a predictable and regular pattern. The SAM arises during embryogenesis. The presumptive cotyledons flank the presumptive SAM in the apical half of the globular embryo. By the heart stage of embryogenesis, the presumptive SAM consists of three layers of cells that are the precursors to the L1, L2 and L3 layers (Barton and Poethig, 1993). As the cotyledons grow out and bend over the top of the embryo (the 'walking-stick stage') these cell layers adopt division patterns characteristic of the SAM. These divisions result in the formation of a small mound of cells with the tunica/corpus organization typical of the angiosperm SAM.

The *Arabidopsis SHOOT MERISTEMLESS (STM)* gene is required for the initiation and maintenance of the SAM. Embryos homozygous for strong loss-of-function mutations in the *STM* gene form cotyledons and other embryonic structures but never establish a population of self-renewing stem cells (Barton and Poethig, 1993). In *stm* mutant embryos, the three precursor layers to the SAM fail to undergo the cell divisions that generate the tunica/corpus organization of the SAM. Thus, the *stm* phenotype is detectable anatomically at the walking stick stage when no mound of cells recognizable as the embryonic SAM is formed. Plants homozygous for weak *stm* alleles form SAMs but these terminate prematurely (Clark et al., 1996; Endrizzi et al., 1996; Felix et al., 1996).

The *STM* gene encodes a Knotted-like homeodomain containing protein and is therefore likely to act as a transcriptional regulator in promoting SAM development (Long et al., 1996). Within the embryo, the *STM* gene is expressed in the presumptive SAM beginning at the late globular stage and expression continues in the developing SAM throughout embryogenesis. Postembryonically, *STM* is expressed throughout the vegetative, inflorescence and floral meristems and is excluded from presumptive organ primordia.

The close correlation between lack of *STM* expression and organ primordium formation suggests that *STM* promotes SAM development by negatively regulating leaf fate and/or positively regulating 'meristem' cell fate within the apex. Here, we explore the development of apical pattern in wild-type and *stm* mutant embryos by analyzing the expression of the *AINTEGUMENTA (ANT)*, *UNUSUAL FLORAL ORGANS (UFO)*, and *CLAVATA1 (CLV1)* genes. In addition to establishing which aspects of apical embryonic pattern *STM* is required for, and which it is not required for, this analysis shows that the apical pattern characteristic of the SAM develops gradually during embryogenesis.

The *ANT*, *UFO* and *CLV1* genes were chosen because their transcripts are limited to subdomains of apical meristems. The *UFO* and *CLV1* subdomains fall within the domain of *STM* expression (Clark et al., 1997; Lee et al., 1997), and the *ANT* gene shows a complementary expression pattern to that of *STM* (Elliott et al., 1996). The *UFO* gene is required for the correct

placement of floral organs and for the correct specification of floral organ fate (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). If *UFO* has a role outside the inflorescence, it must be redundantly encoded by another gene since *ufo* mutants exhibit normal embryonic and vegetative development. The *CLV1* gene is required to limit SAM size (Leyser and Furner, 1992; Clark et al., 1993) and is expressed in the L3 and possibly the L2 layers of the SAM (Clark et al., 1997). The *ANT* gene is expressed in presumptive organs throughout the plant, including the cotyledons (Elliott et al., 1996). In the flower, *ANT* plays a role in the outgrowth of sepals, stamens and petals. However, since the mutant phenotype of *ant* homozygotes is limited to the flower (Elliott et al., 1996; Klucher et al., 1996), if *ANT* has a role in embryonic or vegetative development, this role must be redundantly performed by another gene.

MATERIALS AND METHODS

Growth conditions and genetics

Soil-grown plants were grown in Metromix 200 (Grace Sierra Co.) under continuous light (100 microeinsteins/m²/second) in Conviron growth chambers at 24°C.

stm-1 is a strong mutant allele and has been described previously (Barton and Poethig, 1993; Long et al., 1996). The *stm-11* allele is due to a nonsense mutation at codon 196. In all characters tested, i.e. extent of cotyledon fusion and rate of escape, *stm-11* is a stronger mutant allele than *stm-1* and thus more closely approximates a null allele.

In situ hybridization

In situ hybridization was performed as described by Long et al. (1996). A detailed protocol is posted at <http://www.wisc.edu/genetics/CATG/barton/index.html>.

Double labeling: To detect both *STM* and *UFO* transcripts in the same section, antisense *STM* transcripts were labeled with digoxigenin-11-UTP and antisense *UFO* transcripts were labeled with fluorescein-12-UTP. The two probes were hybridized in approximately equimolar amounts. *STM* transcripts were detected first using anti-digoxigenin antibody (diluted 1:1250) to which alkaline phosphatase had been conjugated (Boehringer Mannheim). Fast Red was used as the substrate. The slides were then mounted in water, photographed and placed in 1× SSC for 2.5 hours at 65°C to deactivate the alkaline phosphatase. Slides were placed in 1× NTE for 10 minutes and blocked in 1% BSA in 100 mM Tris 7.5, 150 mM NaCl, 0.3% Triton X-100 for 45 minutes. *UFO* transcripts were detected using anti-fluorescein antibodies (Boehringer Mannheim) conjugated to alkaline phosphatase (diluted 1:6000 and incubated at room temperature for 2 hours). Western Blue was used as the substrate, slides were mounted in GelMount (Fisher Scientific) and rephotographed.

Probes: Antisense *STM* transcripts were made from meriHB1, a 1.1 kb cDNA clone as described by Long et al. (1996). Antisense *UFO* transcripts were made from pDW22.1 as in Lee et al. (1997). For *ANT*, a 516 base pair fragment (corresponding to nucleotides 613 to 1129 of the *ANT* coding region (Klucher et al., 1996)) was amplified from Landsberg *erecta* genomic DNA using PFU polymerase (Stratagene) and the following primers: ANT1 – 5'ATATATCTCG-AGGGTGGAGGATTTCTTTGGGACCC3' and ANT2 – 5'ATATATTCTAGAAACGCCTCGGTATTGAGAAGTTGCG-3'. This fragment does not contain the AP2-like repeats found in *ANT* to prevent crosshybridization to other AP2 like genes. For *CLV1*, two probes were used which gave identical results. The first probe contained a 1206 base pair fragment (corresponding to nucleotides 48 to 1254 of *CLV1*; Clark et al., 1997) using the primers CLV1a – 5'AGACTCGAGTCTCACTGAGAGGACAC3' and CLV1b –

5'AGAATCTAGAGTTAGAGAGAATTAAC3'. The second probe contained a 326 base pair fragment (corresponding to nucleotides 2787 to 3113) using the primers CLV1c – 5'ACACCTCGAGTTCGGAGTGGTTTTGTTGG3' and CLV1d – 5'ATCTTCTAGACATATTTATCTTGCTTGGG3'.

Stages of embryogenesis

Globular stage embryos are spherical with more than 16 cells. Transition stage embryos are no longer spherical but the developing cotyledons do not yet extend above the embryonic apex. In heart stage embryos the developing cotyledons extend above the apex but make up less than one half of the length of the embryo. In torpedo stage embryos, the cotyledons make up about half of the embryo length. Walking-stick embryos are those in which the cotyledons bend over the apex.

RESULTS

The pattern of *STM* expression differs in central and peripheral regions of the embryo

The *STM* gene is expressed in the SAM progenitor cells beginning at the late globular stage and continuing throughout embryogenesis (Long et al., 1996). Its expression is confined to the presumptive SAM and excluded from the presumptive cotyledons. However, both the histological analysis of SAM formation and the analysis of *STM* expression were confined primarily to the longitudinal, frontal plane of the embryo. (See Fig. 1 for definition of section planes.) We have extended the analysis of *STM* expression in the developing embryo to the sagittal and transverse planes. All of the analysis presented is based on the observation of serial sections of complete embryos.

STM mRNA is found in a continuous band between the cotyledons of the heart-shaped embryo (Fig. 2C-F). This band of expression begins in the globular embryo where it is found first in the peripheral regions and subsequently enlarges to include the central regions. Expression is first detected in one or two cells in the globular stage embryo; these cells are always slightly displaced from the center of the embryo (Fig. 2A). Subsequently, the domain of *STM* expression enlarges such that expression is detected on both sides of the globular/transition stage embryo (see sagittal section in Fig. 2B). At this stage, the peripheral

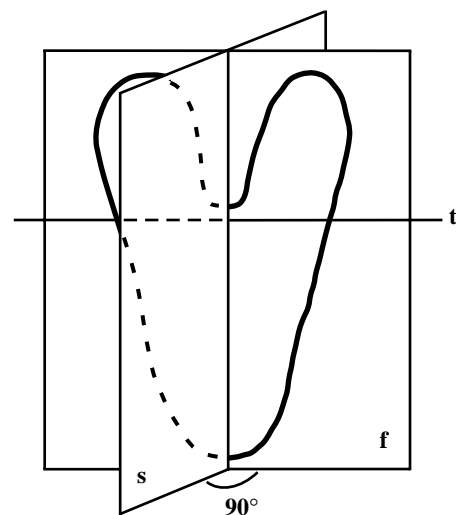


Fig. 1. Definition of section planes through embryo. f, frontal longitudinal; s, sagittal longitudinal; t, transverse.

regions of the presumptive SAM express detectable levels of *STM* mRNA, but the central region does not. By the early heart stage of embryogenesis, expression has spread and is found in a continuous band between the presumptive cotyledons (Fig. 2C,D). We will refer to the peripheral regions of *STM* expression as the embryonic peripheral region (EPR) and the central region as the embryonic central region (ECR).

The observation that expression of *STM* in the peripheral regions of the embryonic SAM can be separated from that in the central regions of the embryonic SAM shows that differences in radial positional information are present within the presumptive SAM at this early stage and that *STM* expression is sensitive to these differences. Sections behind and in front of that shown in Fig. 2B contain the presumptive cotyledons and lack *STM* expression. In the hundreds of globular embryos we have examined, we have never observed *STM* expression in the entirety of the apical half of the globular embryo. Thus, cotyledons and leaves differ in the type of cells from which they derive; all of the cells of true leaves originate from cells that express *STM*, but the bulk of the cotyledons originate from cells that have never expressed *STM*.

The band of *STM* expression persists through the heart stage but as the embryo advances through the torpedo and the walking-stick stages of embryogenesis, expression in the EPR decreases relative to the expression in the ECR (Fig. 2F,H,J). By the time the embryo is fully developed, expression is reduced or undetectable in the EPR but remains strong in the ECR (Fig. 2K,L). The depth of *STM* expression in the EPR and the ECR also differ. While expression in the ECR is confined to the region above the *o'* boundary, expression in the EPR in the heart and torpedo stage embryos extends below the *o'* boundary into the basal half of the embryo (Fig. 2F,H). (The *o'* boundary is formed at the eight cell stage of embryogenesis and divides the embryo proper into upper and lower halves.)

In addition to having different *STM* expression patterns, the EPR and the ECR differ in their ultimate fates. Cells in the ECR participate in making the meristematic dome that forms between the cotyledons in the wild-type embryo, while cells in the EPR participate in forming the separations between the cotyledons. This can be seen in the wild-type seedling in Fig. 3. The two cotyledons wrap around the apex and meet at their bases in the periphery of the medial sagittal plane. Since plant cells do

not migrate, the cells in the valleys where the cotyledons meet must derive from the EPR. In agreement with this, the bases of the cotyledons meet along a region that is three to four cells in diameter in the wild-type – approximately the same number of cell files found in the EPR at the torpedo stage (Fig. 2H).

In *stm* seedlings, the meristematic dome fails to form from

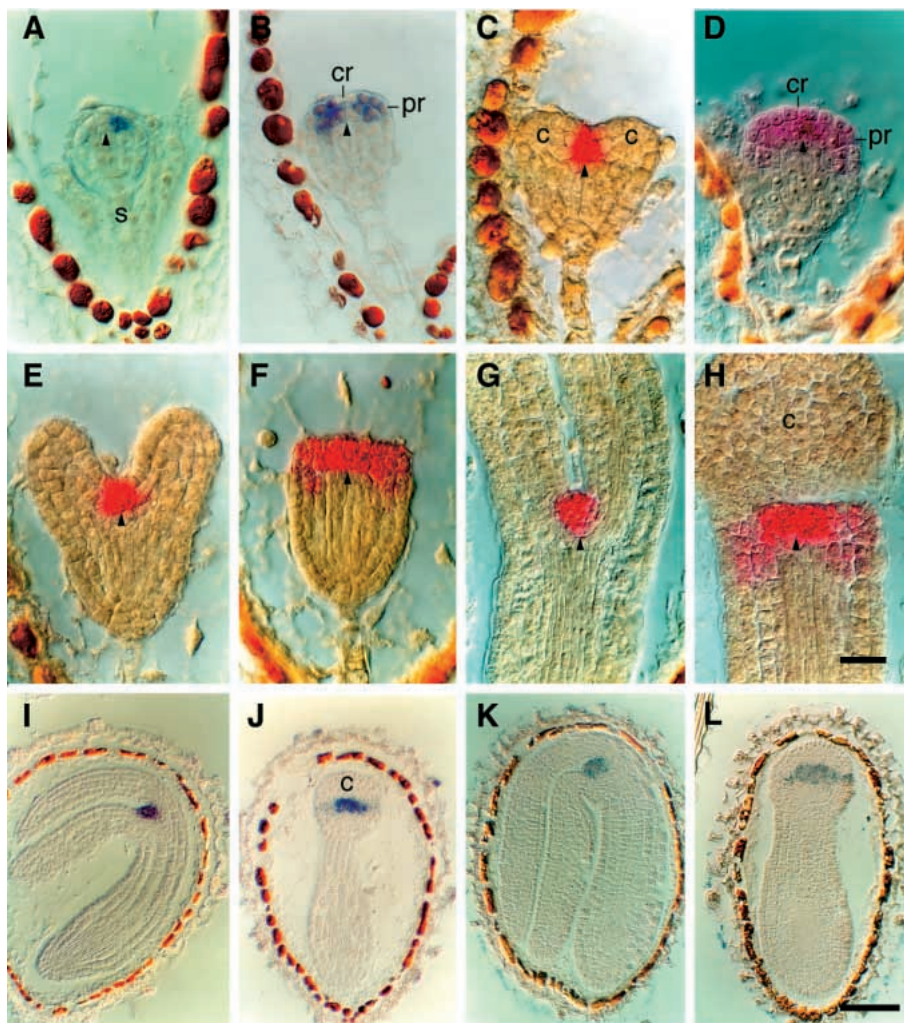


Fig. 2. Pattern of accumulation of *STM* transcript over the course of embryonic development. (A) Sagittal section through late globular embryo showing the first indications of *STM* expression in a single, off center cell. Serial sections through this embryo and others of similar age show that expression is indeed confined to a single cell. (B) Sagittal section through a late globular/early transition stage embryo showing *STM* expression in lateral regions of the presumptive SAM. Sections in front of and behind the plane of the page do not show *STM* expression, confirming that expression is confined to a growing band between the cotyledon primordia. (C) Frontal section through early heart stage embryo showing *STM* expression in region between the developing cotyledons that will develop as the SAM. (D) Sagittal section through an early heart stage embryo showing that *STM* expression now extends as a solid band between the cotyledon primordia. (E) Frontal section through a late heart stage embryo. (F) Sagittal section through a late heart stage embryo. (G) Frontal section through a torpedo stage embryo. (H) Sagittal section through a torpedo stage embryo. *STM* expression in the EPR is lower than that in the ECR. (I) Frontal section through a late walking-stick stage embryo. (J) Transverse section through the SAM of a late walking-stick stage embryo. (K) Frontal section through a fully developed embryo. (L) Transverse section through the SAM of a fully developed embryo. The substrate used was Western Blue in A,B and I-L and Fast Red in p C-H. pr, embryonic peripheral region; cr, embryonic central region; arrowhead, *o'* boundary; c, cotyledon; s, suspensor. Scale bars: H (A-H) 25 μ m; L (I-L) 100 μ m.

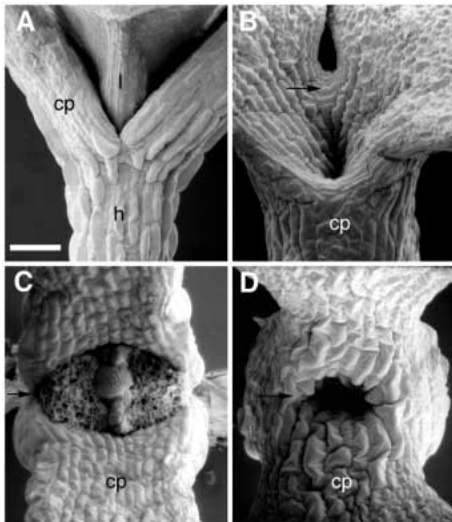


Fig. 3. Scanning electron micrograph of wild-type and homozygous *stm-11* mutant seedlings at 7 days postgermination. (A) Wild-type seedling (*Landsberg erecta*) viewed from the side. True leaves, derivatives of the SAM, are apparent at the apex of the wild-type seedling. The bases of the cotyledon petioles join at an acute angle. (B) Homozygous *stm-11* seedling viewed from the side. There is a gap between the cotyledons where the SAM would normally be. The cotyledon petioles are fused (arrow). (C) Wild-type seedling (*Landsberg erecta*) viewed from above. Arrow indicates the three to four files of cells that form the 'valleys' between the cotyledons. (D) Homozygous *stm-11* seedling viewed from above. Arrow indicates fused region of cotyledon petiole. h, hypocotyl; cp, cotyledon petiole; l, true leaf, scale bar, 150 μ m.

the ECR and the cotyledon petioles are often fused (Fig. 3; Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996). The interdigitation of cells in the fused regions indicates that the fusion is due to outgrowth of the region normally responsible for separation rather than to post-genital fusion. If *STM* acts cell autonomously, *STM* function in the ECR is required for the meristematic dome to form while *STM* function in the EPR is required to prevent outgrowth at the junction of the cotyledon bases. Thus one simple interpretation of *STM* function, that it is required to promote cell division in the SAM, holds only for the ECR. The opposite is true in the EPR where *STM* is required to prevent cell division.

Effect of *STM* on apical embryonic pattern

Since the *STM* gene encodes a predicted homeodomain containing protein, it likely functions by regulating, either positively or negatively, the transcription of other genes. We have determined when and in what pattern three genes expressed in vegetative apices, the *UFO*, *CLV1* and *ANT* genes, are expressed in the embryo during apical development. We have also determined whether their expression requires *STM*.

UFO expression in wild-type and *stm* embryos

The *UFO* gene is expressed in vegetative, inflorescence and floral meristems where it is found at low levels in the center of the SAM and at higher levels towards the periphery of the SAM (Lee et al., 1997). We have examined the expression of *UFO* in the embryo and find that it is limited to a subset of *STM*-

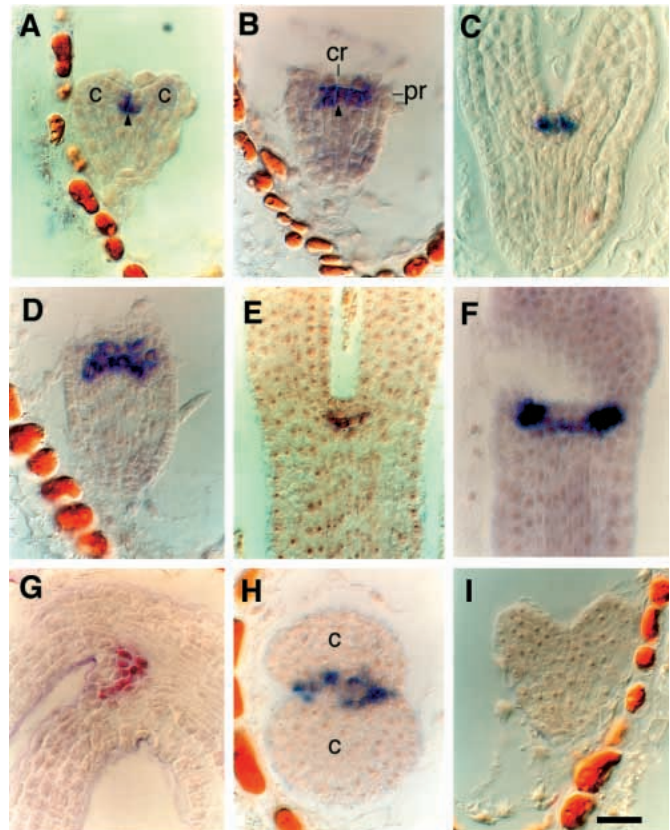


Fig. 4. Pattern of accumulation of *UNUSUAL FLORAL ORGANS* transcript over the course of embryonic development. (A) Frontal section through early heart stage embryo. *UFO* expression first appears in the presumptive L2 and L3 layers around this stage. (B) Sagittal section through early heart stage embryo. Unlike *STM*, *UFO* expression is restricted to central regions of the developing SAM. (C) Frontal section through early torpedo stage embryo. (D) Sagittal section through early torpedo stage embryo. *UFO* expression may extend into presumptive L1 layer at this stage. (E) Frontal section through a late torpedo stage embryo showing *UFO* expression in the form of a cup that surrounds the developing SAM. (F) Sagittal section through a walking-stick stage embryo. *UFO* is still expressed in a cup-shape in the SAM. In addition, two strongly expressing regions are present. (G) Frontal section through a walking-stick stage embryo. (H) Transverse section through a torpedo stage embryo showing a region lacking *UFO* expression in the center of the meristem. (I) Frontal section through an early heart stage *stm-11* embryo hybridized to a probe for *UFO*. No *UFO* expression was seen in any sections of this embryo. Note that the morphology of this embryo is very similar to the wild-type embryo in Fig. 5C. The substrate used was Western Blue in all panels except E and G where it was Fast Red. pr, embryonic peripheral region; cr, embryonic central region; arrowhead, o' boundary; c, cotyledon. Scale bar, 25 μ m.

expressing cells and is dynamic during embryogenesis. *UFO* transcripts are first found in a small group of cells at the presumptive apex at the early heart stage (Fig. 4A,B). *UFO* transcripts are limited to a subset of cells in the *STM* expression domain; they do not accumulate in the EPR (Fig. 4B). *UFO* expression at this early stage is limited to the presumptive L2 and L3 layers and does not extend into the L1 (or outer) layer of the embryo. *UFO* expression becomes more intense in the

late heart/ early torpedo stage embryo where it is expressed between the cotyledons and may extend into the presumptive L1 (Fig. 4C,D). Toward the torpedo stage of embryogenesis, *UFO* expression resolves into a cup-shaped domain at the base of the ECR (Fig. 4E-H). In the fully developed embryo, a faint cup-shaped region of *UFO* expression persists around the SAM (Fig. 4G). In addition, two brightly staining regions of *UFO* expression are detectable at the outer boundaries of the SAM in sagittal section (Fig. 4F).

To determine the extent of overlap between the domains of expression of *UFO* and *STM*, labelling for both genes was done on the same section. In both heart and torpedo stage embryos, the domain of *UFO* expression falls entirely within the domain of high *STM* expression we call the ECR (Fig. 5). In later stages, we were not able to determine the degree of overlap of *UFO* and *STM* expression due to lower levels of *UFO* mRNA in older embryos.

The temporal and spatial pattern of *UFO* expression make it a candidate for positive regulation by *STM*. To test this, we assayed *UFO* expression in self-progeny embryos from parents of genotype *stm-1/+* or *stm-11/+*. 29/122 mid-heart stage embryos from an *stm/+* parent failed to express *UFO* (Fig. 4I). This is consistent with the 25% *stm* embryos expected in these siliques. (Siliques were cut in half, fixed and embedded. The

embryos counted were from a total of 12 siliques and three separate in situ hybridization experiments. All sections of each embryo scored were examined). In wild-type controls (six separate in situ hybridization experiments in which greater than 200 embryos were examined), we never observed embryos at this stage that failed to express *UFO*. At later stages, when *stm* embryos could be detected morphologically, we find that they also fail to express *UFO*. We conclude that *STM* is required for the expression of *UFO* and that the *STM* gene product is active by the early heart stage of embryo development. Note that at the early heart stage of embryogenesis, the morphology of the *stm* embryo is indistinguishable from that of wild-type, so we expect that the cells normally expressing *UFO* in the wild-type embryo are present in the *stm* mutant embryo.

***CLV1* expression in wild-type and *stm* embryos**

The *CLV1* gene, like the *UFO* gene, is expressed in vegetative, inflorescence and floral meristems where it is found in the central region in the L3 and possibly the L2 layers (Clark et al., 1997). We have examined its expression in the embryo and find that it is expressed in the L2 and L3 layers of the developing SAM beginning at the heart stage of embryogenesis (Fig. 6A,B). This is later than both *UFO* and *STM* are first detected. Early heart stage embryos, such as the one in Fig. 6A, exhibited light and variable staining. By late heart stage, expression was detected in all wild-type embryos examined ($n=150$; data collected from 3 separate in situ hybridization experiments). *CLV1* expression, as seen in transverse section, is limited to a subset of cells in the ECR (Compare Fig. 6C with D). As embryogenesis proceeds, it remains expressed in the ECR (Fig. 6E,F,G,H).

In late heart to torpedo stage embryos from self-fertilized *stm-11/+* plants (i.e. embryos between stages shown in Fig. 6B and E), 95 exhibited wild-type *CLV1* expression, 19 showed severely reduced expression and expression was absent in 8. (The same result was seen in three separate in situ hybridization experiments. The data were collected from the experiment that gave the strongest signal.) Together the reduced expression and absent expression categories of embryos make up 22% of total embryos. Later in embryonic development, embryos that were morphologically distinguishable as *stm* mutants exhibited very low or no *CLV1* expression (Fig. 6I,J,K). The embryos shown in Fig. 6J,K were chosen to illustrate the domain of *CLV1* expression in *stm* mutants but are atypical in the high level of expression detected. We conclude that the onset of *CLV1* expression during embryogenesis does not absolutely require *STM* and that *STM* is required for *CLV1* expression to be maintained at wild-type levels. Note that whereas the effect of the *stm* mutation on *UFO* transcript accumulation is observed before any detectable morphological defect is seen in *stm* mutant embryos, *CLV1* expression is affected around the time *stm* mutant embryos deviate morphologically from wild-type embryos.

In addition to expression in the developing SAM, two small (one or two cells) and transient patches of *CLV1* expression were detected in some heart stage embryos (not shown). These patches were outside the procambium and just below the cotyledons in the medial longitudinal plane.

***ANT* expression in wild-type and *stm* embryos**

The *ANT* gene is expressed in the developing cotyledons in the

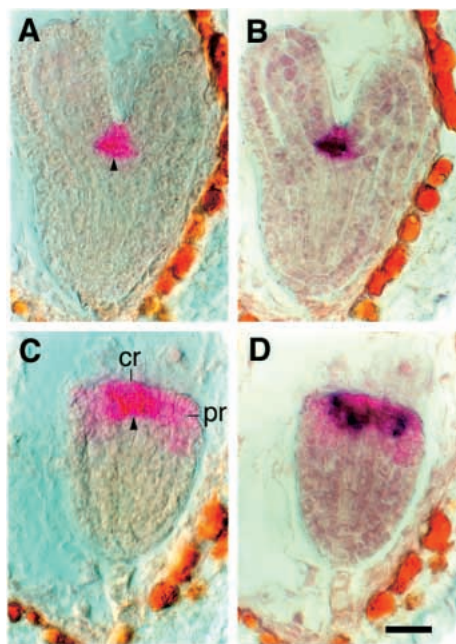


Fig. 5. Simultaneous detection of *UFO* and *STM* transcripts in wild-type embryos. In this experiment *STM* expression is detected first as a red precipitate, and *UFO* expression is detected in a subsequent reaction as a blue precipitate that overlies the red precipitate. (A) Frontal section through an early torpedo stage embryo showing *STM* expression in red. (B) The same section hybridized to a probe for *UFO* (blue precipitate). The domain of *UFO* expression falls entirely within that for *STM* expression. (C) Sagittal section through torpedo stage embryo showing *STM* expression in red. (D) The same section as in C hybridized to a probe for *UFO* (blue). The domain of *UFO* expression falls entirely within that for *STM* expression. pr, embryonic peripheral region; cr, embryonic central region; arrowhead, o' boundary. Scale bar, 25 μ m.

embryo and excluded from the presumptive SAM, thus showing a near opposite pattern of expression to that of *STM* (Elliott et al., 1996). *ANT* is first detected in the 32 cell stage globular embryo where it is found in a few cells in the apical portion of the embryo. We find that *ANT* expression forms a ring in the apical portion of the embryo by the late globular to transition stage of embryogenesis (Fig. 7A,B). Thus, the pattern of *STM* and *ANT* expression in the embryo is not exactly complementary since the domains overlap in the EPR. The ring-shaped pattern of *ANT* expression in the late globular to transition stage embryo is consistent with the expression of this gene in presumptive organs since the cotyledons wrap around and encircle the apical portion of the embryo as shown in Fig. 3. *ANT* expression continues in the heart stage embryo (Fig. 7C,D). As the cotyledons grow out, the region of *ANT* expression becomes progressively limited to a plane that separates the cotyledons into near equal ad- and abaxial regions (Elliott et al., 1996 and our observations).

ANT is excluded from the ECR. If *STM* is required to negatively regulate *ANT* in the ECR, we would expect to see the region of *ANT* expression expand into the ECR in *stm* mutant embryos. However, in 133/133 self-progeny embryos from parents of genotype *stm-11/+*, we detected no changes in the expression pattern of *ANT* and conclude that negative regulation of *ANT* in the ECR is not dependent on *STM* activity. In addition, since *STM* and *ANT* transcripts coexist in the EPR, the presence of *STM* transcript alone is not sufficient to negatively regulate *ANT* expression.

DISCUSSION

Establishing molecular domains in the apical portion of the early embryo

Our results show that pattern develops stepwise in the apical portion of the *Arabidopsis* embryo (Fig. 8). The *ANT* gene is expressed in a peripheral ring and is excluded from the center of the late globular/early transition stage embryo. The pattern of *ANT* expression, including its exclusion from the central region of the embryo, does not require *STM*. *STM* expression, first detected slightly later than *ANT* expression, is expressed in a stripe across the apical half of the transition/early heart stage embryo and divides the apical portion of the embryo into two bilaterally symmetrical halves. *UFO* transcripts appear in the presumptive SAM at the early heart stage of development and require *STM* for their accumulation. By the torpedo stage of embryogenesis, *UFO* expression resolves into a cup-

shaped domain within the SAM. *CLVI* transcripts appear first at the heart stage in a small number of cells in the center of the SAM and do not require *STM* for their accumulation.

Our experiments suggest a model for embryonic apical development in which presumptive cotyledon fate is specified in a ring-shaped domain in the late globular embryo. This is followed by the development of bilateral symmetry, one aspect of which is the specification of the presumptive SAM. As the presumptive SAM develops, it becomes progressively divided into additional domains.

Four observations indicate that radial information (i.e. signals that distinguish central regions of the embryo from peripheral regions) is present in the apical portion of the globular embryo and does not require *STM* function. First, the 'ring' pattern of *ANT* expression, including the exclusion of its transcript from the ECR, is not dependent on wild-type *STM* function; it is found prior to the first detectable *STM* expression and is not altered in *stm* mutant embryos. Second, the *STM* gene itself is expressed in a temporal pattern in the radial dimension: *STM* is present first in the EPR and only

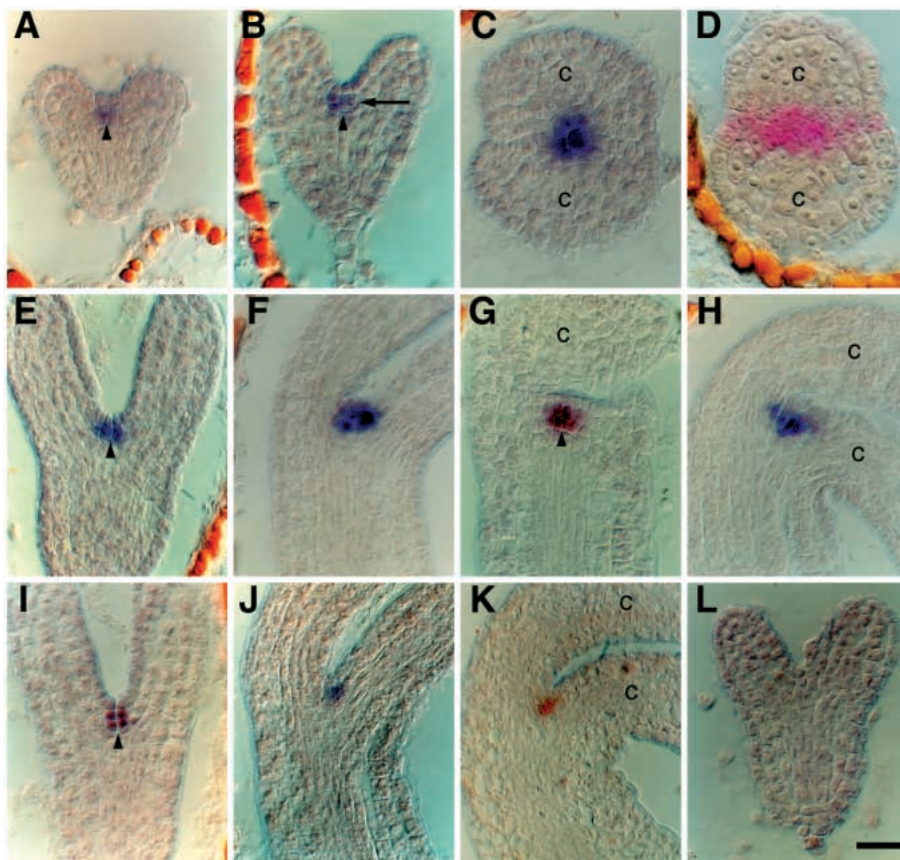


Fig. 6. Expression of *CLVI* RNA in wild-type and *stm* mutant embryos. (A) Wild-type early heart stage embryo. *CLVI* RNA is detected in a few cells in the L2 and L3 layers of the developing SAM. (B) Wild-type late heart stage embryo. (C) Transverse section through a late heart stage embryo. The section is taken at the level shown by the horizontal arrow in B. (D) Transverse section through a late heart stage embryo showing *STM* expression for comparison. (E) Wild-type torpedo stage embryo. (F) Wild-type walking-stick stage embryo. (G) Sagittal section through wild-type walking-stick stage embryo. (H) Wild-type late stage embryo. (I) Torpedo stage *stm-11* embryo. (J) Walking-stick *stm-11* embryo. (K) Late stage *stm-11* embryo. (L) Late heart stage embryo probed with sense strand probe. c, cotyledons; arrowhead, o' boundary. Scale bar, 25 μ m.

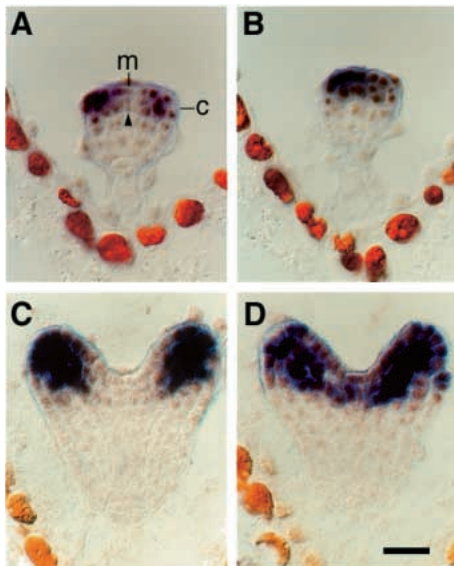


Fig. 7. Expression of *AINTEGUMENTA* mRNA in transition and heart stage embryos. (A) Frontal longitudinal section through a wild-type transition stage embryo. *ANT* mRNA is found in the presumptive cotyledons and is missing from the presumptive SAM. (B) Adjacent section to that shown in A. In more peripheral regions, *ANT* is expressed across the entire apical half of the embryo. (C) Frontal longitudinal section through a wild-type heart stage embryo. *ANT* mRNA remains in the cotyledon primordia and is excluded from the presumptive SAM. (D) Section adjacent to that shown in C. *ANT* mRNA is expressed throughout the apical portion of the embryo. c, presumptive cotyledon; m, presumptive SAM; arrowhead, o' boundary. Scale bar, 25 μ m.

subsequently in the ECR. Third, in the absence of *STM* function, the EPR behaves differently from the ECR — cells in the ECR appear to differentiate without dividing while cells in the outer region divide extensively, ultimately developing as cotyledon petiole. Fourth, the expression of *CLVI* in the centralmost region of the developing SAM does not require *STM*.

In addition to exhibiting different cell fates along the radial axis, *Arabidopsis* embryos develop with bilateral symmetry. The *STM* expression pattern is an early molecular indication of bilateral symmetry in the embryo. There are several cases in which a lack of bilateral symmetry is correlated with a failure to form a SAM. For instance, *cup-shaped cotyledon* mutants (Aida et al., 1997) and *topless* mutants (Long and Barton, unpublished) in *Arabidopsis* frequently lack bilateral symmetry. These mutants fail to form a SAM, and cotyledon tissue develops in the apical region of the embryo in a radially symmetrical pattern. The absence of normal bilateral symmetry may result in an inability to activate the *STM* gene, thus explaining the failure to develop a SAM.

The *STM*-dependent accumulation of *UFO* in the transition/early heart stage embryo indicates that *STM* functions by this stage. It also suggests that *UFO* is a direct target of *STM* activation. Since the expression of *UFO* and *STM* do not correspond in a one-for-one manner, additional factors must act in combination with *STM* to direct *UFO* expression. *UFO* is initially expressed in a block of cells in the

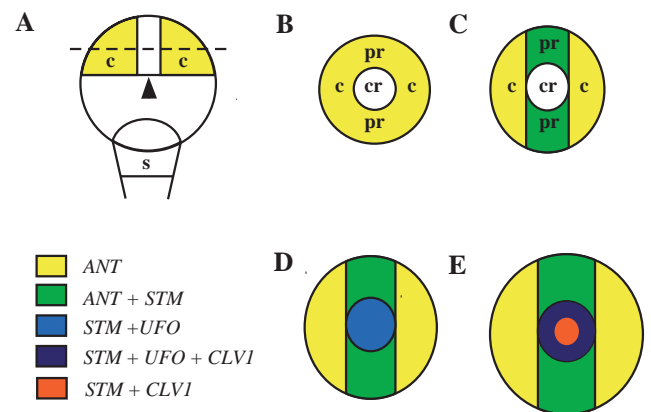


Fig. 8. Schematic of development of apical pattern in the early *Arabidopsis* embryo. (A) The globular stage embryo. *ANT* is expressed above the o' boundary in a ring that includes the presumptive cotyledons and the embryonic peripheral region (EPR) of the presumptive SAM and is excluded from the embryonic central region (ECR) of the presumptive SAM. (B-E) Transverse sections of: (B) the embryo in A at the level of the dashed line — *ANT* appears as a ring of expression which includes the EPR; (C) a transition stage embryo showing the domains of *STM* and *ANT* expression in the EPR but not the ECR; (D) a slightly older embryo showing *STM* as a stripe through the EPR and ECR. *UFO* is expressed in the ECR by the early heart stage; (E) a heart stage embryo showing the domain of *CLVI* expression in the center of the ECR and the domain of *UFO* expression in the ECR. The degree of overlap of *CLVI* with *UFO* is not known. s, suspensor; c, presumptive cotyledon; arrowhead, o' boundary; pr, embryonic peripheral region; cr, embryonic central region.

ECR but, as the ECR grows, becomes limited to a cup-shaped region just inside the limits of the ECR. One possibility is that proximity to the EPR, in combination with *STM*, is required for *UFO* expression.

The function of *UFO* in the embryonic and vegetative SAMs, if any, is unknown. Several functions of a gene expressed at the boundary between primordium and meristem can be imagined. Boundaries of gene expression in other systems can act as sources of morphogens (Cohen, 1996). In addition, the boundary between meristem and leaf is likely to be a site at which cell division is carefully controlled. Support for the latter idea comes from the observation that the *Antirrhinum FIMBRIATA* gene product (*FIM* is the snapdragon ortholog to *UFO*) interacts with proteins implicated in cell cycle control (Ingram et al., 1997).

It has been hypothesized that the apical portion of the globular embryo represents the first incarnation of the SAM (Kaplan and Cooke, 1997). This hypothesis is based chiefly on the chain of logic that since cotyledons and leaves are very similar to each other and since leaves are derived from a SAM, cotyledons must also be derived from a SAM. Several findings presented here and elsewhere (Long et al., 1996) are not consistent with this interpretation. First, cotyledons and true leaves differ in their origin; the majority of the cells from which the cotyledons are derived have never expressed *STM*, while true leaves derive entirely from a lineage of *STM*-expressing cells. Thus, the embryonic cells that give rise to the bulk of the cotyledons never possess the stem cell-like character of giving

rise to both cells like themselves and cells destined to differentiate. Second, several characteristics of the vegetative SAM, both molecular and morphological, are not present in the globular stage embryo. These include the tunica/corpus organization of the SAM and genes expressed in the SAM such as *UFO*, *STM* and *CLVI*. Instead, these characteristics develop gradually during embryogenesis and this program of development requires *STM* for at least some aspects.

The role of *STM* in embryonic SAM function

Different authors have emphasized various, in some cases overlapping, roles for the *STM* gene in SAM function. For instance, Barton and Poethig (1993) concluded that *STM* was required for the initiation of the SAM during embryogenesis because in its absence, no population of stem cells was formed and cells within the presumptive SAM failed to divide and organize into a tunica/corpus organization. The organization of cells into tunica and corpus is a hallmark of angiosperm SAMs and was the only marker of SAM fate available at the time. Meyerowitz (1997) has focused on the requirement of *STM* for cell division in the SAM, since in its absence fewer cell divisions take place. Chuck and Hake (1996) have stressed the requirement for *STM* in preventing differentiation of cells within the SAM. This is similar to the interpretation by Endrizzi et al. (1996), that *STM* is required for the maintenance of an undifferentiated population of cells within the SAM.

Our observations suggest that, even in the relatively simple developing embryo, *STM* functions in a complex manner that can be described adequately only by combining the above concepts. We propose a model in which *STM* acts differently in the embryonic peripheral region (EPR) and in the embryonic central region (ECR). Our model posits that in the EPR, *STM* is required to inhibit organ outgrowth and subsequent differentiation but not organ specification, whereas in the ECR, *STM* is required to inhibit differentiation and to initiate a SAM-specific program of development.

The model is based on the observation that the EPR and the ECR differ in several ways. They differ in the fates of their cells both in the presence and the absence of *STM*, the time of onset and the persistence of *STM* expression, and in the distribution of the *UFO*, *ANT* and *CLVI* transcripts. Let us first consider the EPR. Cells in the EPR are destined to form the separations between the cotyledons. To do so, cells in this region must be restricted in their outgrowth relative to the adjacent regions. In the absence of *STM* function, cells in the EPR form a region of fused cotyledon petiole instead of the normal separation between cotyledons. The morphology of the fused petiole region in *stm* mutants, with its extensively interdigitated cells, is most consistent with the fusion forming by inappropriate division and outgrowth of cells of the EPR. Thus, *STM* is required to inhibit the outgrowth of these cells but not to prevent their specification as part of the cotyledons since *ANT* expression, a marker for organ primordia, in the EPR overlaps with *STM*. Since cells in the EPR ultimately differentiate, *STM* is required to delay their differentiation rather than prevent it indefinitely. Consistent with this, the expression of *STM* in the EPR is transient – decreasing during embryogenesis and ultimately disappearing completely in the germinated seedling (data not shown).

This model requires that the instructions as to what portion of the cotyledon the EPR cells will form, change over time.

Thus, in the complete absence of *STM* activity, cells in the EPR grow out prematurely, and develop as cotyledon petiole. In the wild-type, cells in the EPR do not differentiate until *STM* levels are below some critical level, presumably past the heart stage of development. At this point, the instructions for cotyledon development have changed so that only a more limited basal region of the cotyledon petiole develops. (Note: this is similar to the maturation schedule hypothesis for maize leaf development (Muehlbauer et al., 1997).)

In the ECR, *STM* is required to prevent differentiation. In the absence of *STM* function, cells in the ECR differentiate instead of forming a small mound of cells as they do in the wild-type. This mound of cells includes the ‘stem cells’ that are responsible for the growth and development of the shoot system. As opposed to cells in the EPR, these cells persistently express *STM*. We propose that, in addition to preventing differentiation in the ECR, *STM* is required for the implementation of a SAM-specific program of development in the ECR through the activation of a SAM-specific battery of genes. *UFO* is an example of a SAM-specific gene whose expression depends on *STM*.

Not all gene expression in the developing embryonic SAM requires *STM*; the *CLVI* transcript appears in *stm* mutant embryo apices. However, maintenance of *CLVI* expression at normal levels requires *STM*. With regard to embryonic SAM formation, *stm* is epistatic to *clvi* (Clark et al., 1996). This observation is consistent with two models of gene interaction. In a regulatory model, *CLVI* could negatively regulate *STM*; in the absence of *STM* function, the presence or absence of its negative regulator, *CLVI*, is indistinguishable and the *stm* phenotype is observed. In an assembly model, *STM* acts to promote the development of a tissue, in this case an apical meristem (or some intermediate stage in apical meristem formation). The *CLVI* gene then acts to control the size of this tissue, the apical meristem. In the absence of the tissue, the presence or absence of *CLVI* is indistinguishable and the *stm* phenotype is observed. Our results are consistent with both models and in addition indicate that *STM* is a positive regulator of *CLVI*.

What might explain the difference in action of *STM* in the EPR versus the ECR? The relative levels of *STM* protein may differ in the two regions with lower levels dictating EPR activity while higher levels dictate ECR activity. Alternatively, *STM* may act in combination with other factors whose presence or absence differs in the EPR and the ECR.

Genes such as *PINHEAD/ZWILLE* (McConnell and Barton, 1995; Moussian et al., 1998) and *WUSCHEL* (Laux et al., 1996) which are required for the formation of a functional SAM during embryogenesis may be specifically required for ECR development. Mutants in these genes show normal cotyledon separation and premature SAM termination.

A model for dual *STM* functions helps to explain the two phenotypes seen in plants that over- or ectopically express other *KNOTTED*-like genes. In some cases, overexpression results in the formation of ectopic SAMs on the leaf surface (Sinha et al., 1993; Chuck and Hake, 1996). This is consistent with the conclusion that *KNOTTED*-like genes have the ability to activate a SAM-specific program of development, analogous to the role proposed for *STM* in the ECR. In other cases, ectopic expression of *KNOTTED*-like genes causes increased lobing or division of leaves (Lincoln et al., 1994; Chuck et al., 1996;

Hareven et al., 1996). Lobing can be explained by *KNOTTED*-like genes preventing the growth and differentiation of cells specified as leaf. This is analogous to the role proposed for *STM* in the EPR. Just as in the embryo, the difference in phenotypes between plants that overexpress *KNOTTED*-like genes could result from differences in the level or the context of gene expression.

The model for dual action of *STM* may extend to the peripheral and central zones of postembryonic meristems. The localization of *STM* in the EPR and the fate of cells in this region parallel that in the PZ of the vegetative SAM. *STM* is expressed between developing organ primordia in the vegetative and floral meristems (Long et al., 1996) and is required to prevent organ fusion in flowers (Felix et al., 1996). Likewise, *STM* is expressed in the central zone of vegetative and floral meristems (Long et al., 1996) and is required to prevent premature meristem termination (Clark et al., 1996; Endrizzi et al., 1996).

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