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Shoot apical meristem and cotyledon formation during *Arabidopsis* embryogenesis: interaction among the *CUP-SHAPED COTYLEDON* and *SHOOT MERISTEMLESS* genes

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SUMMARY

The shoot apical meristem and cotyledons of higher plants are established during embryogenesis in the apex. Redundant *CUP-SHAPED COTYLEDON 1* (*CUC1*) and *CUC2* as well as *SHOOT MERISTEMLESS* (*STM*) of *Arabidopsis* are required for shoot apical meristem formation and cotyledon separation. To elucidate how the apical region of the embryo is established, we investigated genetic interactions among *CUC1*, *CUC2* and *STM*, as well as the expression patterns of *CUC2* and *STM* mRNA. Expression of these genes marked the incipient shoot apical meristem as well as the boundaries of cotyledon primordia, consistent with their roles for shoot apical meristem formation and cotyledon separation. Genetic and expression analyses indicate that *CUC1* and *CUC2* are redundantly required for expression of *STM* to form the shoot apical meristem, and that *STM* is required for proper spatial expression of *CUC2* to separate cotyledons. A model for pattern formation in the apical region of the *Arabidopsis* embryo is presented.

Key words: Meristem, Cotyledon, Organ separation, *Arabidopsis thaliana*, *CUP-SHAPED COTYLEDON* (*CUC*), *SHOOT MERISTEMLESS* (*STM*), Pattern formation, Embryogenesis

INTRODUCTION

In higher plants, most of the above-ground part ultimately derives from small populations of mitotic cells, called the shoot apical meristem (SAM). The SAM is initially formed during embryogenesis, when the basic body architecture of a plant is established (Jürgens, 1995). Once formed, the SAM plays central roles in postembryonic shoot organ formation. The SAM generates stems, leaves, and floral organs in a set pattern while it maintains a pool of undifferentiated cells in the center (Steeves and Sussex, 1989). Thus, SAM formation during embryogenesis is a critical step to start subsequent vegetative and reproductive development. Many of the recent molecular genetic works have been focused on SAM function in postembryonic development (reviewed in Clark, 1997; Meyerowitz, 1997). However, the molecular genetic basis of SAM formation during embryogenesis is poorly understood.

The embryonic SAM is formed in the apex between cotyledons in dicotyledonous plants. In *Arabidopsis*, the zygote undergoes stereotyped cell divisions to form the radially symmetrical embryo proper and the extraembryonic suspensor (the globular stage). By the heart stage, cotyledon primordia arise as two distinct bumps from the apical flanks of the embryo and the symmetry of the embryo shifts from radial to bilateral. As cotyledons grow and bend over the embryo apex (the bending-cotyledon stage), the SAM becomes a histologically distinct structure (Barton and Poethig, 1993). Both histological and clonal analyses suggest that the entire SAM and most of the cotyledons derive from the apical half of the globular embryo (Barton and Poethig, 1993; Scheres et al., 1994).

Several *Arabidopsis* mutants are defective only in the SAM, suggesting that at least some part of SAM formation is genetically distinct from that of cotyledons. Recessive mutations in *PINHEAD* (*PNH*; same as *ZWILLE* [*ZLL*], which was identified independently) and *WUSCHEL* (*WUS*) specifically affect SAM formation, resulting in a flat or aberrant structure at the site normally occupied by the SAM (McConnell and Barton, 1995; Laux et al., 1996; Moussian et al., 1998). These genes are suggested to be involved in organizing functional domains within the SAM. The *clavata1* (*clv1*) and *clv3* mutants have an enlarged SAM, suggesting that *CLV1* and *CLV3* are required to limit cell populations within the SAM (Clark et al., 1995, 1996).

On the other hand, several mutations that affect development of both the SAM and cotyledons have been identified, suggesting that their genetic pathways overlap. Mutations in the *SHOOT MERISTEMLESS* (*STM*) gene result in the lack of a SAM and a slight fusion of the cotyledons at the base, indicating that *STM* is essential for embryonic SAM formation and partially required for cotyledon separation (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996; Long and Barton, 1998). Weak alleles of *stm* produce a phenotype

that suggests that *STM* is also required for maintenance of the SAM (Clark et al., 1996; Endrizzi et al., 1996). *STM* encodes a member of the KNOTTED1 class of homeodomain proteins (Long et al., 1996). This class of proteins are thought to be key transcriptional regulators of SAM development and constitute a gene family found in many plants including maize (Kerstetter et al., 1994), rice (Matsuoka et al., 1993), tomato (Hareven et al., 1996), tobacco (Tamaoki et al., 1997), and *Arabidopsis* (Lincoln et al., 1994). *STM* mRNA is expressed in the SAM as well as its precursor cells, consistent with its role in SAM formation and maintenance (Long et al., 1996). Genetic analyses show that the *stm* mutation is epistatic to other SAM-specific mutations, *pnh*/*zll*, *wus*, *clv1* and *clv3*, with regards to embryonic SAM formation (McConnell and Barton, 1995; Clark et al., 1996; Endrizzi et al., 1996), suggesting that *STM* acts upstream of these genes in this process.

Arabidopsis *CUP-SHAPED COTYLEDON 2* (*CUC2*) and petunia *no apical meristem* (*nam*) are members of another class of genes involved in both SAM formation and cotyledon separation (Souer et al., 1996; Aida et al., 1997). Both genes encode members of the NAC domain proteins, whose biochemical function is unknown. Double mutations in *CUC2* and another redundant gene, *CUC1*, cause the lack of an embryonic SAM and a nearly complete fusion of the cotyledons, although each single mutant is basically normal (Aida et al., 1997). Mutations in the *nam* gene cause similar, but weaker defects than the *cuc1 cuc2* double mutant. In *nam* mutants, no SAM develops and cotyledons are partially fused on one side (Souer et al., 1996). Adventitious shoots are occasionally formed from tissue culture of *cuc1 cuc2* mutant hypocotyls or from *nam* mutant seedlings, and these shoots show almost normal vegetative and reproductive development except that their flowers have several defects including organ fusion (Souer et al., 1996; Aida et al., 1997). These observations suggest that the *CUC1*, *CUC2* and *nam* genes are not essential for SAM maintenance. Expression of *nam* is not detected in the SAM itself but in the boundaries of the SAM and cotyledons (Souer et al., 1996). This unique expression pattern, together with the mutant phenotypes of *nam* and *cuc1 cuc2*, indicates a close relationship between SAM formation and boundary specification. However, interaction between the NAC genes and *STM* in these processes remains to be determined.

To investigate roles of *CUC1*, *CUC2* and *STM* during embryogenesis, we examined in detail the temporal and spatial expression of *CUC2* and *STM* mRNAs during embryogenesis. In addition, expression of *CUC2* in the *stm* mutant and expression of *STM* in the *cuc1 cuc2* double mutant were examined. We also examined phenotypes of the double and triple mutants of *cuc1*, *cuc2*, and *stm*. The data indicate that the *CUC1* and *CUC2* are essential for *STM* expression to form the SAM and that *STM* is required for proper spatial expression of *CUC2* to separate cotyledons.

MATERIALS AND METHODS

Plants and growth conditions

Arabidopsis thaliana ecotype Landsberg *erecta* was used as the wild type. The origin of the *cuc* mutants was described previously (Aida et al., 1997). The origins of the *stm-1* and *stm-2* mutants are as described by Barton and Poethig (1993) and Clark et al. (1996). Plants were soil grown at 23°C under constant white light as previously described (Fukaki et al., 1996) and siliques were collected for analyses of embryo phenotypes and in situ hybridization. Stages of embryogenesis are as defined in Jürgens and Mayer (1994). For examination of seedling phenotypes, seeds were surface sterilized, sown on Murashige and Skoog plates, and germinated as previously described (Aida et al., 1997).

Construction of the double and triple mutants

For construction of the double mutants, plants heterozygous for *stm-1* were crossed with *cuc1* or *cuc2* single homozygous mutant plants. Among F_2 populations, plants homozygous for *cuc1* or *cuc2* and heterozygous for *stm-1* were selected based on the floral phenotypes of the *cuc* single mutants (Aida et al., 1997) and PCR analyses which could detect the *stm-1* mutation. Phenotypes of the double mutants were examined in the F_3 generation. For construction of the triple mutants, plants heterozygous for *stm-1* were crossed with plants homozygous for *cuc1* and heterozygous for *cuc2*. Two F_2 families that segregated both *cuc1 cuc2* and *stm-1* mutants were selected (family 1 and 2). In family 1, approx. 1/16 seedlings with the *cuc1 cuc2* double mutant phenotype were observed (16 of 400 F_2s, χ^2=3.46, P>0.05; calculation based on 1:15 ratio of *cuc1 cuc2* seedlings to others), and phenotypes of the other F_2 seedlings were normal, *stm-1*, *cuc1 stm-1*, or *cuc2 stm-1*. Genotypes of *STM* locus in the *cuc1 cuc2* seedlings were examined using PCR analysis with specific primers. Among them, 3 were homozygous for *stm-1*, 10 were heterozygous for *stm-1*, and the remaining 3 were homozygous for the wild-type allele, indicating independent inheritance of the *STM* alleles in *cuc1 cuc2* double mutants. Essentially the same result was obtained in family 2. The results indicate that *cuc1 cuc2 stm-1* shows the same phenotype as that of *cuc1 cuc2*.

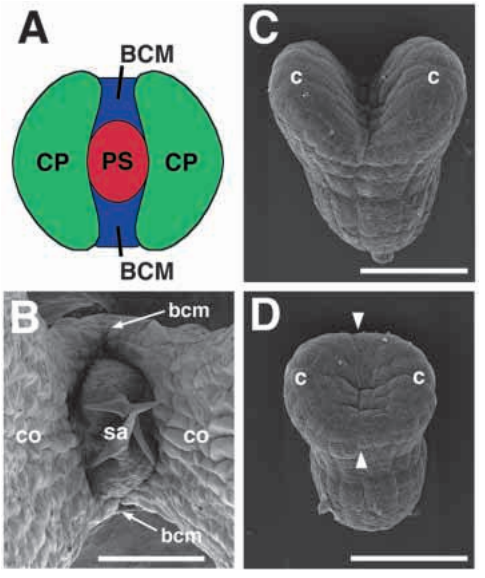
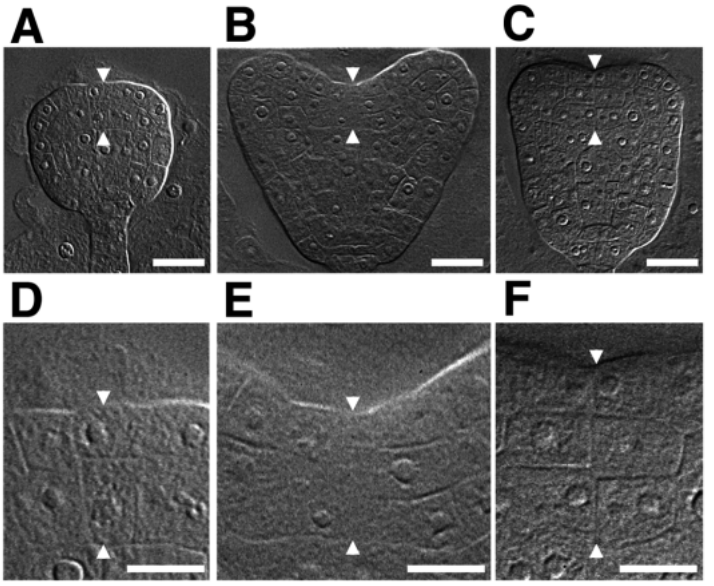


Fig. 1. Development of the apical region in the wild type and *cuc1 cuc2* embryos. (A) Schematic diagram of the apical region of the wild-type embryo viewed from above. CP, cotyledon primordia region; PS, presumptive SAM region; BCM, boundary region of cotyledon margins. (B-D) Scanning electron micrograph (SEM) images of (B) wild-type seedling at 3 days postgermination viewed from above; (C) wild-type embryo at the heart stage; (D) *cuc1 cuc2* embryo at the heart stage. Arrowheads indicate ectopic bulging of BCM. Scale bars, 100 μm (B) and 40 μm (C,D). c, cotyledon primordia; co, cotyledons; sa, SAM; bcm, boundaries of cotyledon margins.

Scanning electron microscopy (SEM)

Seedlings or dissected embryos were fixed in FAA overnight at 4°C. Embryos were attached to poly-L-lysine coated coverslips before the subsequent steps. Samples were then subjected to dehydration and critical point drying. Samples were mounted on stubs and coated with gold in an ion splutter coater before observation.



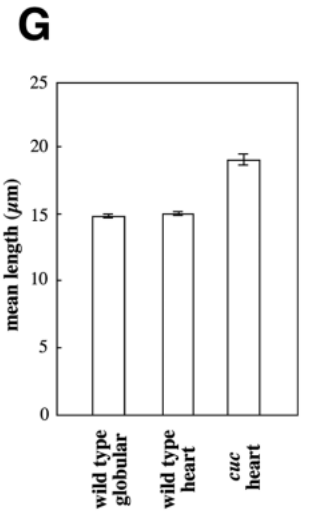


Fig. 2. Ectopic bulging of the presumptive SAM (PS) in *cuc1 cuc2* embryos. Embryos were cleared and viewed with Nomarski optics. Lower arrowheads indicate the O′ line and upper arrowheads indicate the embryo apex. The distance between the arrowheads indicates the length of the PS in the apical-basal direction. (A) Wild-type embryo at the globular stage. (B) Wild-type embryo at the heart stage. (C) *cuc1 cuc2* embryo at the heart stage. (D-F) Higher magnifications of A, B and C respectively. (G) Mean lengths of PS in the apical-basal direction ($n \geq 18$). Error bars represent standard error. Scale bars, 20 μm (A-C) and 10 μm (D-F).

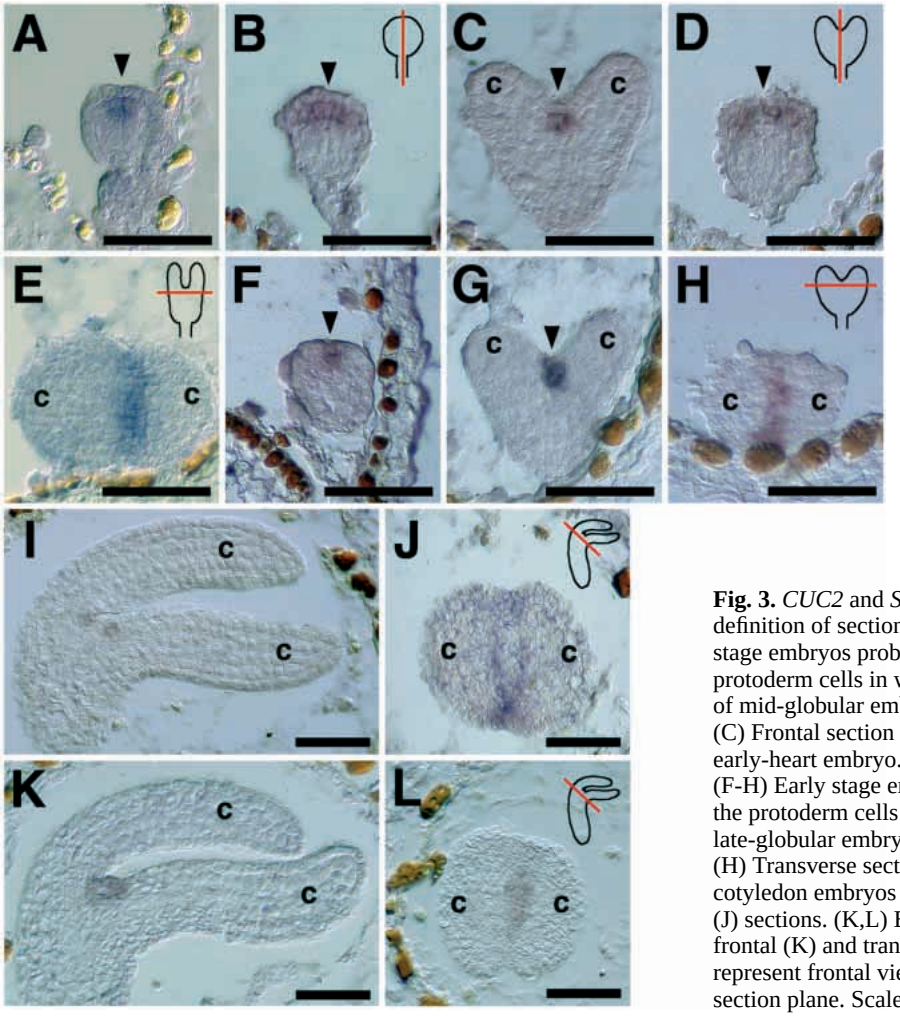


Fig. 3. *CUC2* and *STM* mRNA expression in wild-type embryos. For definition of section planes, see Long and Barton (1998). (A-E) Early stage embryos probed with *CUC2*. Arrowheads indicate the protoderm cells in which *CUC2* is not detected. (A) Frontal section of mid-globular embryo. (B) Sagittal section of late-globular embryo. (C) Frontal section of early-heart embryo. (D) Sagittal section of early-heart embryo. (E) Transverse section of torpedo embryo. (F-H) Early stage embryos probed with *STM*. Arrowheads indicate the protoderm cells in which *STM* is detected. (F) Frontal section of late-globular embryo. (G) Frontal section of early-heart embryo. (H) Transverse section of early heart embryo. (I,J) Bending cotyledon embryos probed with *CUC2* in frontal (I) and transverse (J) sections. (K,L) Bending cotyledon embryos probed with *STM* in frontal (K) and transverse (L) sections. Diagrams in B,D,E,H,J,L represent frontal view of each embryo with red line indicating the section plane. Scale bars, 40 μm. c, cotyledon primordium.

Clearing of embryos and seedlings

For visualization of embryos or seedling vasculature, ovules or seedlings were cleared as previously described (Aida et al., 1997). Nomarski images (in Fig. 2) were processed in Adobe Photoshop so that cell walls could be clearly seen.

In situ hybridization

Digoxigenin-labeled RNA probes were synthesized with in vitro transcription using T3 or T7 RNA polymerase according to manufacturer's instruction (Boehringer Mannheim). Templates for transcription of *CUC2* antisense probes were derived from a PCR-amplified 558 bp fragment corresponding to the 3rd exon (Aida et al., 1997) or a reverse transcriptase PCR-amplified 1140 bp fragment containing the whole coding region of the *CUC2* cDNA (unpublished data). Both probes gave identical results. The 558 bp fragment does not contain the conserved NAC domain to prevent cross hybridization to other NAC-box containing genes. Templates for an *STM* antisense probe correspond to the region that spans amino acids 81-382 and includes the 3′UTR (Long et al., 1996). Control experiments were performed with or without the sense probes of *CUC2* or *STM* made from the above templates, and no signal above background was detected. Tissues were fixed, dehydrated, and embedded as described by Lincoln et al. (1994). 8 μm sections were cut and attached to 3-aminopropyltriethoxysilane-coated slides (MATSUNAMI). Section pretreatment and hybridization were performed according to the method of Lincoln et al. (1994), except that hybridization of *CUC2* probes was performed at 45°C. Immunological detection was performed as described by Coen et al. (1990).

RESULTS

The *cuc1 cuc2* and *stm* mutants show defects in SAM formation and cotyledon separation

The apical region of the wild-type embryo of *Arabidopsis* can be divided into three types of subregions based on their final fates, which become apparent in the seedling (Fig. 1A,B). The first region is bilateral, where cotyledons arise (referred to as CP, cotyledon primordia region). Bulging of cotyledon primordia begins at the heart stage (Fig. 1C). The second region, at the center, gives rise to the SAM (referred to as PS, presumptive SAM region). The PS does not bulge during early stages of cotyledon formation (Fig. 1C), but later at the bending-cotyledon stage, it bulges slightly as the SAM become histologically apparent (Barton and Poethig, 1993). The third is a boundary region of cotyledon margins (referred to as BCM). The BCM does not bulge throughout embryogenesis so that cotyledons are separated from each other.

Both the *cuc1 cuc2* double mutant and *stm* single mutant show defects in PS and BCM. The phenotype of *cuc1 cuc2* was first apparent at the heart stage (Aida et al., 1997). From this stage on, ectopic bulging occurs in the BCM, which leads to the congenital fusion of cotyledon primordia (Fig. 1D). This eventually results in the formation of fused cup-shaped cotyledons (Aida et al., 1997). We found that ectopic bulging occurred not only in the BCM, but also in the PS slightly and transiently during the transition from the globular to heart stages (Fig. 2).

In longitudinal sections, the globular embryo is divided into the apical and basal halves by the O′ line (Fig. 2A, lower arrowhead), which derives from transverse cell divisions at the eight-cell stage (West and Harada, 1993). Above the O' line, cell number in the apical-basal direction of PS is normally two (Fig. 2A,D). Some of the hypodermal cells in this region divide transversely by the heart stage, resulting in two or three cells (Fig. 2B,E; Barton and Poethig, 1993). In spite of an increase in the number of cells, the length of the PS in the apical-basal direction did not changed markedly during the transition from the globular to heart stages (Fig. 2G). In *cuc1 cuc2* heart stage embryos, however, the length was significantly greater compared to the wild type at the same stage (Fig. 2G; $P<0.0001$ Student's *t*-test), although the number of cells was two or three as in the wild type (Fig. 2E,F). These results suggest that the cells in the PS of the *cuc1 cuc2* double mutant elongate abnormally in the apical-basal direction and caused slight bulging in this region. In later heart stages, however, the PS is apparently depressed (Fig. 1D), suggesting that the ectopic bulging of the PS is only transient.

In *stm* embryos, ectopic bulging in the BCM was first detected at the bending-cotyledon stage (data not shown), resulting in slight fusion of cotyledon petioles (Clark et al., 1996; Endrizzi et al., 1996; Long and Barton, 1998). Both *cuc1 cuc2* and *stm* mutants completely lack an embryonic SAM, which is normally formed in the PS (Barton and Poethig, 1993; Aida et al., 1997). Thus, in normal development, *CUC1*, *CUC2* and *STM* are suggested to function in the PS to form the SAM and in the BCM to repress bulging for cotyledon separation, although the contribution of *STM* in the BCM is much smaller than that of *CUC1* and *CUC2*. *CUC1* and *CUC2* also seem to be required for the repression of bulging in the PS during the transition from the globular to heart stages.

Expression patterns of *CUC2* and *STM* in wild-type embryos

To confirm the site where *CUC2* and *STM* function, we performed in situ hybridization in wild-type embryos using *CUC2* and *STM* specific probes. Expression of *STM* during embryogenesis has already been examined in detail (Long et al., 1996; Long and Barton, 1998), and we largely confirmed the previous results.

In early stages of embryo development, the expression of both genes largely overlapped (Fig. 3A-H). The *CUC2* mRNA was detected at the early- to mid-globular stages in a few cells in the PS just above the O′ line (Fig. 3A). By the late-globular stage, the signal spread along the medial axis, resulting in a stripe across the top half of the embryo (Fig. 3B). At the heart stage, the stripe pattern was detected between cotyledon primordia (Fig. 3C,D) and continued until the torpedo stage (Fig. 3E). The *CUC2* signal was restricted to the hypodermal cells and was not detected in the protoderm cells (arrowheads in Fig. 3A-D). *STM* mRNA was first detected slightly later than *CUC2*, in the late-globular stage (Fig. 3F). Similar to *CUC2*, *STM* subsequently spread as a stripe between cotyledons and the stripe continued through the heart stage (Fig. 3G,H) until the torpedo stage (data not shown). Unlike *CUC2*, the *STM* signal was also detected in the protoderm cells (arrowheads in Fig. 3F,G).

Later in the bending-cotyledon stage, when the developing SAM began to bulge slightly, spatial expression of the two genes became different (Fig. 3I-L). *CUC2* was not detected at the center, and it was detected in a region surrounding the SAM (Fig. 3I,J). This region corresponds to BCM and the boundaries between the SAM and cotyledons. The *CUC2* signal was detected in the protoderm cells, unlike in the early stages. In

contrast to *CUC2*, *STM* signal disappeared from BCM, and was detected only in the SAM (Fig. 3K,L). Whether the signals of *CUC2* and *STM* overlap or not is unclear.

STM expression requires *CUC1* and *CUC2*

To determine the relationship between *CUC1*, *CUC2* and *STM*, we constructed the triple mutant of *cuc1*, *cuc2* and *stm-1* (see Materials and methods). The seedling phenotype of the triple mutant was the same as that of the *cuc1 cuc2* double mutant, showing fused cup-shaped cotyledons and complete lack of an embryonic SAM (Fig. 4A,B). This epistasis of *cuc1 cuc2* to *stm* suggests that *STM* is not functional in the *cuc1 cuc2* double mutant. Thus, we next examined expression of *STM* in the *cuc1 cuc2* double mutant embryos developing in siliques of *cuc1*/*cuc1 cuc2*/+ plants.

The phenotype of the *cuc1 cuc2* double mutant is first apparent at the heart stage. At this stage, the depression between cotyledon primordia of the double mutant is less clear compared to wild-type embryos (compare Fig. 2B and C). When the *STM* probe was hybridized to *cuc1*/*cuc1 cuc2*/+ siliques, the signal was not detected in the double mutant embryos (Fig. 5A), although it was detected in the other phenotypically normal siblings (Fig. 5B). 20 of 75 heart stage embryos from *cuc1*/*cuc1 cuc2*/+ siliques failed to express *STM*, consistent with the expected ratio of the double mutant embryos. *STM* expression after the heart stage of the double mutant was not detected, either (data not shown). In normal siblings in *cuc1*/*cuc1 cuc2*/+ siliques, intensity and spatial pattern of the *STM* signal was normal (Fig 5B), suggesting that the *cuc1* single mutation did not affect expression of *STM*. Normal expression of *STM* was also observed in *cuc2* single mutant embryos (data not shown). These results indicate that *CUC1* and *CUC2* are redundantly required for *STM* expression.

Double mutants of *cuc* and *stm*

We next constructed and examined the double mutants of *cuc1 stm* and *cuc2 stm* (see Materials and methods). Wild-type seedlings have separate cotyledons (Figs 1B, 4C). Each single mutant of *cuc1* or *cuc2* is basically normal, except that a few seedlings (less than 1%) show cotyledon fusion along one side of the margins (Aida et al., 1997). *stm-1* (strong mutant allele; Long et al., 1996) shows partial cotyledon fusion at the bases (Fig. 4D). The fused region is slightly expanded. Vascular bundles in the expanded region split in two as in wild-type petioles (compare Fig. 4F,G), confirming that this region was fused petioles and not hypocotyl. Severity of fusion somewhat varied among each *stm-1* seedling. Even in the most severe mutant phenotype, however, fusion was restricted to the region from the base to the middle part of the petioles and did not extended to the blades. Symmetry of fusion also varied – extent of fusion was more severe on one side in some seedlings.

Severity of the fusion phenotype was enhanced in *stm-1 cuc2* double mutants compared to that in *stm-1* single mutants (Fig. 4E,H). In the most extreme cases, fusion extended to cotyledon blades. The *cuc1* mutation also enhanced the fusion phenotype of *stm-1*, although the extent of fusion was slightly milder than that of *stm-1 cuc2* (Fig. 4I). We also examined double mutants of *stm-2* (weak mutant allele; Clark et al., 1996) and *cuc1*, as well as *stm-2* and *cuc2*. *stm-2* single mutants show no or only slight fusion of cotyledon petioles (Fig. 4J). The fusion phenotype of *stm-2* was enhanced by both *cuc1* (data not shown) and *cuc2* (Fig. 4K). The extent of fusion was weaker compared to *stm-1 cuc1* or *stm-1 cuc2*.

The synergistic interactions between *cuc1* or *cuc2* and *stm* mutations suggest that *CUC1*/*CUC2* and *STM* act in the same or an overlapping pathways for the separation of cotyledons. To test this further, we next examined expression of *CUC2* in *stm* mutant embryos.

CUC2 spatial expression late in embryogenesis is disturbed in *stm-1* mutants

The *stm-1* mutant phenotype becomes apparent at the bending cotyledon stage (Barton and Poethig, 1993). *CUC2* expression in the *stm-1* mutants before this stage appears to be normal, since we could not detected any abnormal expression in a population of heart stage embryos developing in siliques of plants heterozygous for *stm-1* (n=30, data not shown).

In the bending-cotyledon stage, however, spatial expression of *CUC2* was variable in *stm-1* mutants (compare Figs 3J, 5C, 5D). Fig. 5C shows an example of the *CUC2* expression pattern in a transverse section of an *stm-1* embryo. In this case, *CUC2* signal was detected as a spot in the center. By examining the serial sections cut along transverse or longitudinal planes of different embryos, we observed not only this type but also other types of patterns in which the signal was detected as a single spot in a lateral position (Fig. 5D, top) or two spots in opposite positions between cotyledons (Fig. 5D, bottom). These results indicate that *STM* is required for the proper spatial expression of *CUC2* at the bending-cotyledon stage.

DISCUSSION

Expression of *CUC2* marks the boundaries of cotyledons and the SAM

Expression patterns of *CUC2* and *STM* are schematically represented in Fig. 6. In early stages of embryogenesis, *CUC2* is expressed in PS and BCM, in which ectopic bulging occurs in the *cuc1 cuc2* double mutant. Later, *CUC2* expression is excluded from the center but continues in BCM. These results suggest *CUC2* acts to repress bulging in the boundary between cotyledons to separate them. *CUC2* is also expressed in the boundaries between the developing SAM and cotyledons, suggesting that it is required for repression of bulging in these regions to separate the SAM and cotyledons.

In the *cuc1 cuc2* double mutant, ectopic bulging in the PS is only slight and transient compared to that in the BCM. How this difference occurs is unclear. There may be another factor(s) which specifically represses bulging in the PS. It is also possible that cells in the PS may not be fated to be incorporated into cotyledon primordia per se, whereas cells in the outer region (CP and BCM) may have potential to become cotyledon cells. The different responses of cells in PS and BCM to loss of *CUC1* and *CUC2* activities suggest that a prepattern distinguishing PS from the other regions exists in the apical part of the globular embryo, and the prepattern is not dependent on *CUC1* and *CUC2* (see below).

Interaction of *CUC1*, *CUC2* and *STM* in SAM formation

STM mRNA is expressed in both PS and BCM in the early

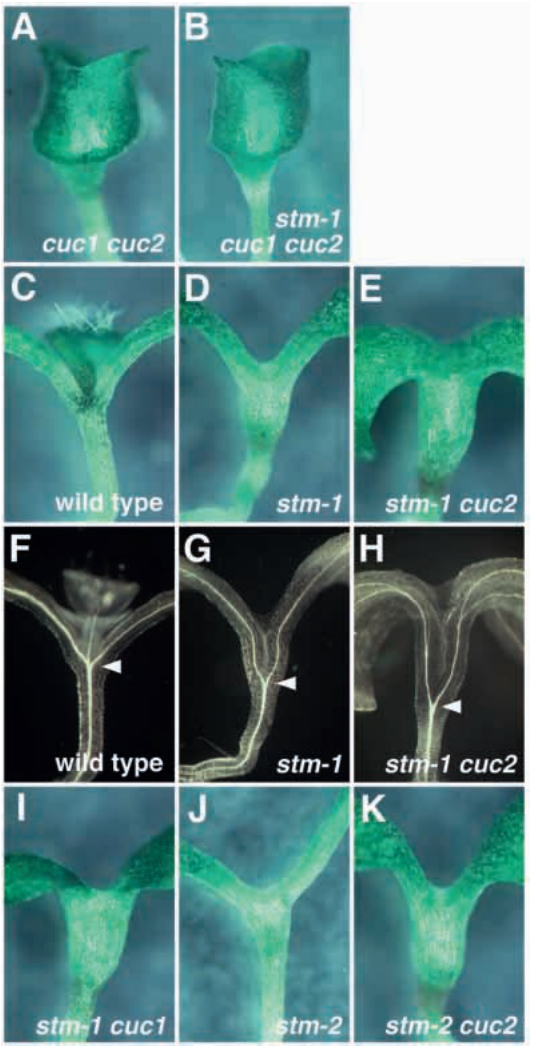


Fig. 4. Double and triple mutants of *cuc1*, *cuc2* and *stm*. Seedlings at 6 days postgermination of *cuc1 cuc2* (A), *stm-1 cuc1 cuc2* (B), wild type (C), *stm-1* (D), *stm-1 cuc2* (E), *stm-1 cuc1* (I), *stm-2* (J), and *stm-2 cuc2* (K). (F,G,H) Cleared seedlings of C,D and E, respectively. Each arrowhead indicates a point where vascular bundles split in two, marking the boundary between hypocotyl and cotyledon petioles.

stages of embryo development, but later, the signal disappears in BCM and becomes restricted to the developing SAM (Fig. 6). This expression pattern is consistent with the *STM* function deduced from the mutant phenotype; i.e., *STM* is mainly required for SAM formation and partially required for cotyledon separation.

Our results indicate that *CUC1* and *CUC2* act upstream of *STM* and are redundantly required for *STM* expression. Loss of *STM* expression in *cuc1 cuc2* embryos accounts for the meristemless phenotype of this mutant. We propose that embryonic SAM formation in *Arabidopsis* is divided into two steps. First, *CUC2* (and presumably *CUC1*) expression occurs in the stripe region of the globular embryo. Secondly, *STM* is activated directly or indirectly by *CUC1* and *CUC2* in this region and starts the SAM-specific program as a transcriptional regulator in the PS.

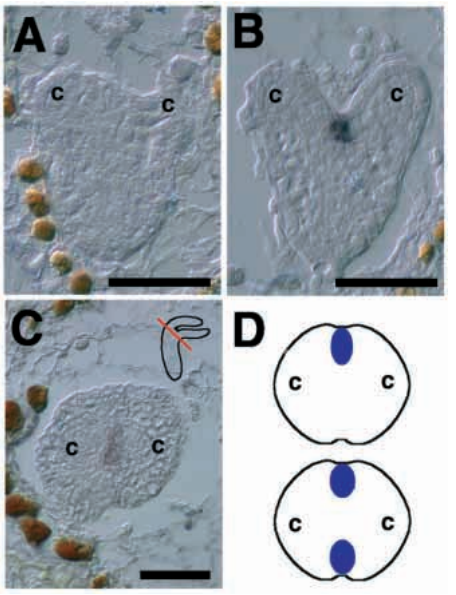


Fig. 5. *STM* expression in *cuc1 cuc2* and *CUC2* expression in *stm-1* embryos. (A) Frontal section of heart stage *cuc1 cuc2* embryo in a *cuc1*/*cuc1 cuc2*/+ silique probed with *STM*. (B) Frontal section of normal siblings in the same silique probed with *STM*. (C) Transverse section of *stm-1* embryo at the bending-cotyledon stage probed with *CUC2*. (D) Schematic diagram of two other variations of *CUC2* expression in transverse sections of *stm-1* embryos at the bending-cotyledon stage. Signals are represented by blue color. Diagram in C represents frontal view of an embryo with the red line indicating the section plane. Scale bars represent 40 μm. c, cotyledon primordium.

Later at the bending-cotyledon stage, *CUC2* expression is downregulated in the developing SAM whereas *STM* is continuously expressed. This observation suggests that, once *STM* is activated, *CUC2* expression in PS is no longer required for the maintenance of *STM* expression at least after the torpedo stage. Whether the downregulation of *CUC2* is essential to accomplish SAM formation is unknown.

How does *CUC2* activate *STM*? One possibility is that the CUC2 protein may directly activate *STM* transcription. There is an implication that at least one NAC domain protein, AtNAM, can act as a transcriptional activator in yeast (M. Duval and T. L. Thomas, personal communication). Alternatively, *CUC2* may affect *STM* expression indirectly through changing expression of other unidentified factors.

Interaction of *CUC1*, *CUC2* and *STM* in cotyledon separation

The synergistic interactions between *cuc2* and *stm* as well as abnormal spatial expression of *CUC2* in *stm* bending-cotyledon embryos suggest that *STM* contributes to cotyledon separation by regulating the spatial pattern of *CUC2* expression late in embryogenesis. Loss of *CUC2* expression at the BCM in *stm* mutants at the later stage of cotyledon development accounts for the partial fusion phenotype of cotyledon petioles. The variable *CUC2* spatial expression may be correlated with the variable fusion phenotypes observed in *stm* seedlings. The phenotype of *cuc1 stm* double mutant seedlings suggests that *STM* also regulates *CUC1* to separate cotyledons.

It is unclear how *STM* regulates the spatial expression of

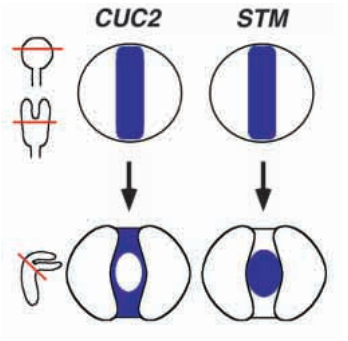


Fig. 6. Schematic diagram of *CUC2* and *STM* mRNA expression patterns. Expression patterns of *CUC2* and *STM* in transverse sections are shown in blue. At the left, the section planes are represented by red lines on frontal views of embryos. (Top) *CUC2* and *STM* expression at early stages (from the globular to torpedo stages). (Bottom) *CUC2* and *STM* expression at the bending-cotyledon stage.

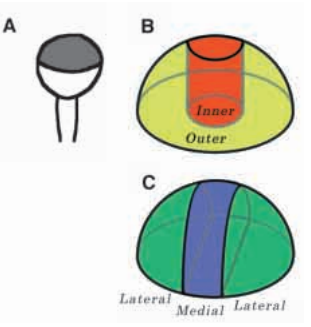


Fig. 7. A model for apical pattern formation. (A) Frontal view of the globular embryo. Apical region is shown in gray. (B) The radial pattern. (C) The bilateral pattern. See text for details.

CUC2 at the bending-cotyledon stage. Considering the nearly complementary distributions of each transcript at this stage, one could suppose mutual repression; i.e., *STM* downregulates *CUC2* in the center and *CUC2* downregulates *STM* in the surrounding region. This simple model, however, does not seem to be appropriate, because loss of *STM* activity results in a variable spot(s) of *CUC2* expression rather than a mere stripe, which is predicted by the model. The variable expression patterns of *CUC2* in *stm* suggest that *STM* may affect *CUC2* expression through a more complex process in which other unidentified factors are involved. Such factors may include negative regulators which downregulate *CUC2* in the center in response to an *STM*-dependent positional cue.

Like *CUC2*, spatial expression of *STM* in the bending-cotyledon stage must be regulated so that downregulation in BCM occurs correctly. Whether this downregulation requires the *CUC* genes is unknown.

A model for apical pattern formation during *Arabidopsis* embryogenesis

The apical region of the embryo consists of three types of subregions, which respectively give rise to cotyledons, the SAM, and cotyledon boundaries. Based on our results, we propose a model that these subregions are established by superimposition of two patterns, radial and bilateral (Fig. 7). The radial pattern consists of the inner and outer regions. This pattern is indicated by the different fates of cells in PS and the surrounding region (CP and BCM) in the *cuc1 cuc2* double mutant; i.e., cells in the surrounding region are incorporated into the bulging cotyledon primordia whereas cell in the center are not. The radial pattern is also indicated by expression of the *AINTEGUMENTA* gene, which is found as a ring in the outer region but not in the inner region of the globular embryo (Long and Barton, 1998). The bilateral pattern consists of the medial region and two equivalent lateral regions. This pattern is indicated by the initial stripe patterns of *CUC2* and *STM* in the globular embryo.

In this model, the radial pattern specifies fates of the inner and outer regions of the globular embryo, so that the outer region can bulge out as cotyledon primordia but the inner region cannot. On the other hand, *CUC2* and *STM* expression begins in the medial region in response to positional information based on the bilateral pattern. Then, *CUC2* represses bulging in the BCM, which corresponds to the overlap of the medial and outer regions. Later in the bending-cotyledon stage, *CUC2* is downregulated in the inner region and so is *STM* in the outer region in response to positional information based on the radial pattern.

How these patterns are established is not yet known. In *cuc1 cuc2* double mutants and *stm* mutants, the PS does not markedly bulge in contrast to the surrounding regions, suggesting that the radial pattern is retained. The stripe of *CUC2* expression is still observed in *stm* mutants, suggesting that the mutation does not affect the bilateral pattern. Histological analysis indicates that bilateral symmetry is retained in the *cuc1 cuc2* double mutant (Aida et al., 1997). Thus, *CUC1*, *CUC2* and *STM* may not be required for the formation of either of the patterns. In *stm* mutants, however, sites in which downregulation of *CUC2* occurs is often disturbed. This observation suggests that, in *stm*, the radial pattern is not maintained or positional information based on the radial pattern is not correctly converted to the *CUC2* expression pattern.

Mutations in *PIN-FORMED* (Goto et al., 1991; Liu et al., 1993; Okada and Shimura, 1994), *PINOID* (Bennett et al., 1995), *ALTERED MERISTEM PROGRAM* (Chaudhury et al., 1993), *MONOPTEROS* (Berleth and Jürgens, 1993), and *TOPLESS* (Evans and Barton, 1997) result in altered bilateral symmetry, so that these genes may be involved in establishment of the bilateral pattern. The ring-shaped expression of the *ANT* gene in the globular embryo (Long and Barton, 1998) may suggest its involvement in the radial pattern formation. Detailed analyses of these genes and their relationship to *CUC1*, *CUC2* and *STM* may help to elucidate the origin of the apical patterns.

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