ERRATUM

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On page 3972 of this article, the sentence “This raises the possibility that the effect of *STM* on *CUC1* activity starts much earlier.” should read “This raises the possibility that the effect of *STM* on *CUC1* activity starts much earlier than previously thought.”

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Roles of *PIN-FORMED1* and *MONOPTEROS* in pattern formation of the apical region of the *Arabidopsis* embryo

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SUMMARY

In dicotyledonous plants, the apical region of the embryo shifts from radial to bilateral symmetry as the two cotyledon primordia develop on opposite sides of the shoot meristem. To further elucidate the mechanisms regulating this patterning process, we analyzed functions of two *Arabidopsis* genes, *PIN-FORMED1* (*PIN1*) and *MONOPTEROS* (*MP*), encoding a putative auxin efflux carrier and a transcription factor thought to mediate auxin signaling, respectively. The corresponding mutants show similar defects in apical patterning, including cotyledon fusion and dissymmetric organ positioning. Both mutations perturb the spatial expression patterns of *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2*, which are redundantly required for cotyledon separation and meristem formation. During early embryogenesis, both *CUC* genes are affected differently: the area of *CUC1* expression is expanded while that of *CUC2* expression is reduced. In addition, genetic analysis indicates that *PIN1* and *MP* are required for the activity of *CUC2* while *CUC1* activity is only slightly affected by both mutations. These results suggest a differential regulation of the *CUC* genes by *PIN1* and *MP*. Furthermore, genetic analysis suggests that *SHOOT MERISTEMLESS* (*STM*), another regulator for cotyledon separation and meristem formation, promotes *CUC1* activity in parallel with *PIN1*. Our results suggest a model where *PIN1* and *MP* regulate apical patterning partially through the control of *CUC* gene expression.

Key words: Embryogenesis, Pattern formation, *PIN1*, *MP*, Auxin, *Arabidopsis thaliana*

INTRODUCTION

During embryogenesis of higher plants, only a basic body plan is established including one or two cotyledons, a hypocotyl and a root along the apical-basal axis (Steeves and Sussex, 1989). Two populations of stem cells, the shoot and root meristems, are formed at opposite ends of this axis. These meristems, in turn, will initiate the postembryonic tissues and organs. The establishment of the different embryonic regions is essential for proper post-embryonic development.

From the globular to heart stages of dicotyledonous embryogenesis, the shoot apex of the embryo is partitioned into three subregions, which will give rise to cotyledons, shoot apical meristem (SAM) and cotyledon boundaries (Aida et al., 1999; Bowman and Eshed, 2000; Long and Barton, 1998). The SAM is located at the center and surrounded by two cotyledons, which are initiated in opposite positions. Owing to the symmetrical positioning and size of the cotyledons, the overall morphology of the embryo is bilaterally symmetric.

Several genes of *Arabidopsis* are important for the development of the apical region of the embryo (Aida and Tasaka, 2001; Bowman and Eshed, 2000). Among them, the *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2* genes encode highly homologous, putative transcription factors of the NAC family (Aida et al., 1997; Aida et al., 1999; Takada et al., 2001). The two genes are functionally redundant and required for both SAM initiation and suppression of growth at the cotyledon boundaries. When both of these genes are disrupted, ectopic growth occurs at the boundary, resulting in almost completely fused cotyledons surrounding the apex, suggesting a role of these genes in promoting organ separation at the boundaries. In addition, the *cuc1 cuc2* double mutant does not develop a SAM (Aida et al., 1997). In agreement with their function, the two genes are expressed at the presumptive SAM and cotyledon boundaries during the early heart stage, forming a band that extends between the incipient cotyledons (Aida et al., 1999; Takada et al., 2001). Another important factor is the *SHOOT MERISTEMLESS* (*STM*) gene, which encodes a putative transcription factor of the KNOTTED1 class of homeodomain proteins (Long et al., 1996). The *stm* mutant lacks a functional meristem and shows partially fused cotyledons (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996). *STM* mRNA is detected at the embryo summit from the globular stage onwards, first in a few cells, later on in a stripe covering the SAM and cotyledon boundaries

(Long and Barton, 1998; Long et al., 1996). The mutant phenotype, together with the expression pattern of the gene, shows that *STM* has a major role in SAM initiation and is also implicated in cotyledon separation. Genetic and gene expression studies have revealed important interactions between the *STM* and the *CUC* genes. *STM* is not expressed in the *cuc1 cuc2* double mutant embryo, indicating that *CUC1* and *CUC2* are required for activation of *STM* expression (Aida et al., 1999). Moreover, ectopic expression of *CUC1* induces ectopic *STM* expression, associated with adventitious SAM formation on the surface of cotyledons, indicating that *CUC1* is an upstream regulator of *STM* in SAM formation (Takada et al., 2001). In turn, *STM* is required for proper expression patterns of *CUC1* and *CUC2* during later stages of embryogenesis (Aida et al., 1999; Takada et al., 2001).

Besides these relatively well characterized factors, a limited number of other genes have also been implicated in the patterning of the embryonic shoot apex. In particular, *PIN-FORMED1* (*PIN1*) and *MONOPTEROS* (*MP*) have profound effects on cotyledon development. During embryogenesis, the *pin1* mutation affects cotyledon positioning, number, growth and separation, resulting in disruption of bilateral symmetry (Bennett et al., 1995; Okada et al., 1991). Mutations in the *MP* gene strongly perturb embryo development, affecting the establishment of the embryo axis. In addition, *mp* mutants also display defects in cotyledon positioning and these cotyledons are frequently fused, as observed in *pin1* mutants (Berleth and Jürgens, 1993). Together, these results suggest important roles for *PIN1* and *MP* genes in patterning the apical region of the embryo, possibly via interactions with *CUC1* and *CUC2*.

Interestingly, both *PIN1* and *MP* are linked to the plant hormone auxin. The *PIN1* gene encodes a protein with homologies to a large family of transmembrane transporters (Chen et al., 1998; Friml et al., 2002a; Friml et al., 2002b; Gälweiler et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). The corresponding mutant shows strong reduction in polar auxin transport along the inflorescence stem and there is convincing evidence that *PIN1* encodes an auxin efflux transporter (reviewed by Palme and Gälweiler, 1999). The *MP* gene encodes a member of the AUXIN RESPONSE FACTOR (ARF) gene family (Hardtke and Berleth, 1998). The proteins of this family are proposed to bind functionally defined promoter elements of auxin-inducible genes (Ulmasov et al., 1997). *MP* could thus regulate downstream genes in response to auxin signals.

To further elucidate the mechanism that regulates apical patterning in the *Arabidopsis* embryo, we investigated the roles of *PIN1* and *MP*, especially with regard to their relationship with *CUC1*, *CUC2* and *STM*. Combining a genetic approach with expression studies, we show that *PIN1* and *MP* participate in apical patterning, partially through regulating the expression of *CUC1* and *CUC2*. In addition, our results suggest that *STM* has an important role in organ separation via the regulation of *CUC1* activity.

MATERIALS AND METHODS

Plant strains

The *Arabidopsis thaliana* ecotypes Landsberg *erecta* (L*er*) and Wassilewskija (WS) were used in this study. The following mutant alleles were used: *cuc1-1* (L*er*) (Takada et al., 2001), *cuc2* (L*er*) (Aida et al., 1997), *pin1-3* (L*er*) (Bennett et al., 1995), *pin1-6* (WS) (Vernoux et al., 2000), *stm-dgh6* (WS) and *mp-rtl* (L*er*). *pin1-3* has previously been described as a strong allele (Bennett et al., 1995) and contained a point mutation from GT to AT at the 5′ end of the second intron (M. A. and M. T., unpublished results). This could prevent removal of the intron and lead to a truncated protein. *pin1-6* has been described phenotypically as a strong allele (Vernoux et al., 2000). *stm-dgh6* was identified in the T-DNA collection of Versailles (Bechtold et al., 1993). This allele is supposed to be null as the T-DNA is inserted between nucleotide 723 and 724 in the cDNA sequence. The insertion disrupts the C-terminal end of the protein and eliminates the homeodomain (J. Dockx and J. T., unpublished results). The *mp-rtl* allele was originally described as a *rootless* mutant (Barton and Poethig, 1993). Based on phenotypic criteria described by Berleth and Jürgens (Berleth and Jürgens, 1993), it was classified as a weak allele. The allelism was confirmed by crossing *mp-rtl* to the *mp-T370* and *mp-G92* alleles. *mp-rtl* contained a nonsense mutation in codon 594 (M. A. and M. T., unpublished results).

Growth conditions

Plants were grown on soil at 23°C under constant white light as previously described (Fukaki et al., 1996a) and siliques were collected for analyses of embryo phenotypes and in situ hybridization. Alternatively, plants were grown under greenhouse conditions. Stages of embryogenesis are as defined previously (Jürgens and Mayer, 1994). For examination of seedling phenotypes, seeds were surface sterilized, germinated either on Murashige and Skoog plates as described previously (Fukaki et al., 1996b) or on Arabidopsis medium (Santoni et al., 1994).

Construction of the double and triple mutants

For construction of the *pin1 cuc1 cuc2* triple mutant, plants heterozygous for *pin1-3* or *pin1-6* were crossed with plants homozygous for *cuc1-1* and heterozygous for *cuc2*. F_2 plants homozygous for *cuc1* and heterozygous for both *cuc2* and *pin1* were selected and self-fertilized. For the cross between *cuc1 cuc2* and *pin1-3*, the F_3 seedlings were genotyped using PCR primers that detected the *cuc1*, *cuc2* and *pin1-3* mutations. 12 of 83 seedlings were triple mutants and they all showed essentially the same phenotype. Triple mutants resulting from the cross with *pin1-6* also showed the same phenotype. For construction of the double mutants, plants heterozygous for *pin1-3*, *pin1-6* or *mp-rtl* were crossed with homozygous *cuc1-1* or *cuc2*. Among F_2 populations, plants homozygous for *cuc1* or *cuc2* were selected by their floral phenotype (Aida et al., 1997) and confirmed by PCR-based genotyping. These plants were further selected for the heterozygous *pin1* or *mp-rtl* mutations. Seedling phenotypes were examined in the F_3 generation. The genotypes of *pin1 cuc1* and *pin1 cuc2* double mutants were confirmed either by PCR or the pin-shaped inflorescence phenotype (Okada et al., 1991). The genotypes of *mp cuc1* and *mp cuc2* were confirmed by the rootless phenotype (Berleth and Jürgens, 1993). For the construction of double mutants between *pin1-6* and *stm-dgh6*, crosses between the corresponding heterozygotes were made. Double mutants could be recognized in the segregating F_2 population as plants with a new phenotype.

Microscopy

For visualization of seedling vasculature, plants were cleared as described previously (Aida et al., 1997). Scanning electron micrographs were obtained as described by Aida et al. (Aida et al., 1999). The SAM in mature embryos was visualized by confocal laser scanning microscopy as described previously (Clark et al., 1995). For histological sections, seedlings were fixed in 4% formaldehyde and 1% glutaraldehyde in phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) and embedded in Historesin (Leica) following standard procedures. 5-10 μm sections were made

with a Leica 5010 microtome using steel disposable knives and viewed under a Nikon microscope after staining with Toluidine Blue.

In situ hybridization

In situ hybridization was performed as previously described (Aida et al., 1999) with the following modifications. Embryos were fixed in 4% paraformaldehyde and 4% dimethyl sulfoxide in PBS. A treatment with hydrochloric acid during the prehybridization was omitted. Alternatively, in situ hybridization was performed using the procedure described by Laufs et al. (Laufs et al., 1998). Probes for detecting the following genes have been reported previously: *PIN1* (Gälweiler et al., 1998), *CUC1* (Takada et al., 2001) and *CUC2* (Aida et al., 1999).

RESULTS

Phenotypes of *pin1*, *cuc1 cuc2* and *pin1 cuc1 cuc2*

In a wild-type seedling of *Arabidopsis*, two cotyledons with equal size and shape are arranged symmetrically at the apex (Fig. 1A). They are completely separated from base to top and flank the SAM, which is located between their bases. Each cotyledon has a similar set of vascular bundles, which consists of a single mid-vein running along the center and several lateral veins (Fig. 1B). The origin of this symmetry can be traced back to embryogenesis, when cotyledons are initiated (Fig. 1I). To investigate the roles of *PIN1*, *CUC1* and *CUC2* in the establishment of symmetry, we first re-examined the phenotypes of *cuc1 cuc2* and *pin1* mutants.

The single mutants of *cuc1* and *cuc2* are indistinguishable from wild type except for a few seedlings whose cotyledons are fused on one side (Table 1) (Aida et al., 1997). In all *cuc1 cuc2* double mutant seedlings, however, cotyledons are strongly fused at both margins and surrounded the apex completely (Fig. 1C; Table 1). Nevertheless, several observations suggested that they still showed bilateral symmetry, based on two morphological criteria. First, the uppermost margin of a cup-shaped structure always had two splits marking the cotyledon boundaries, which were positioned symmetrically (Fig. 1C, arrowheads). These splits divided the cup-shaped structure into two equivalent parts with equal size and shape, and were already apparent at the torpedo stage of embryogenesis (Fig. 1J, arrowheads). Second, each of the two parts divided by the splits showed essentially the same vascular pattern that consisted of one mid-vein and two lateral veins (Fig. 1D). This pattern was very similar to the one observed in wild type (Fig. 1B). These results indicate that the *cuc1 cuc2* double mutation strongly disrupts the separation of cotyledons without affecting the bilateral symmetry.

In *pin1*, cotyledons were generally reduced in length compared to wild type. Although the *pin1* seedlings often had two completely separated cotyledons that were arranged symmetrically (Fig. 1E), a significant proportion of the mutants showed defects in cotyledon separation (Table 1). In this sub-population, cotyledon number varied from one to three and the size of cotyledons was often variable even in a single seedling (Fig. 1F). Frequently, adjacent cotyledons were fused to different extents (Fig. 1F, arrowheads). In contrast to *cuc1 cuc2*, however, cotyledons of *pin1* usually remained completely separated at least at one boundary so that the fused cotyledons never surrounded the entire apex. When two cotyledons were fused, their relative positions were always affected so that they were located closer to each other (Fig. 1F, arrowheads). The cotyledon defects in *pin1* were already apparent in torpedo stage embryos, where cotyledons with unequal sizes were initiated at asymmetrical positions (Fig. 1K). These observations indicate that *pin1* mutations disrupt bilateral symmetry and cotyledon separation from early

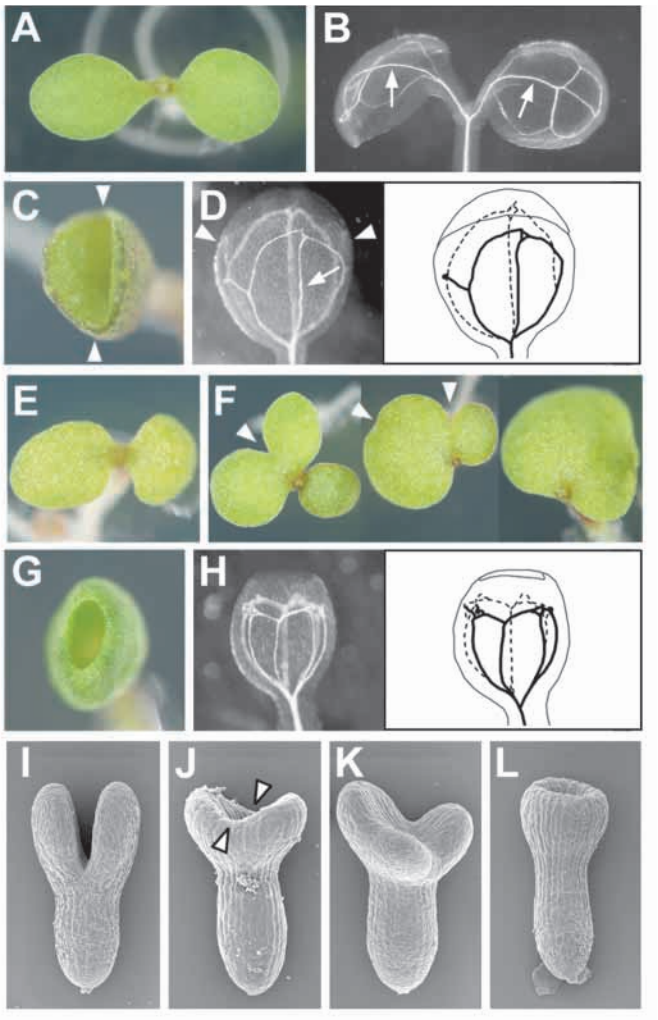

Fig. 1. Phenotypes of wild type, *cuc1 cuc2*, *pin1* and *pin1 cuc1 cuc2*. (A-H) 4-day-old seedlings of wild type (A,B), *cuc1 cuc2* (C,D), *pin1-3* (E,F) and *pin1-3 cuc1 cuc2* (G,H). In B,D and H, seedlings were cleared to visualize the vascular pattern. A wild-type seedling has two symmetrically arranged, completely separated cotyledons (A) and each cotyledon contains a single mid-vein (B, arrows). *cuc1 cuc2* has two bilaterally symmetrical cotyledons, as revealed by two splits at the top (arrowheads in C,D) and a vascular pattern similar to wild type (D). Arrow in D indicates one of the two mid veins (the other one is out of focus). *pin1* shows variable phenotypes including two completely separated cotyledons (E), partial fusion and increased number of cotyledons (F, left and center) and a wide collar-shaped cotyledon (F, right). The fused part is indicated by arrowheads. *pin1 cuc1 cuc2* shows a radially symmetrical morphology as revealed by complete cotyledon fusion (G) and evenly distributed vascular bundles (H). (I-L) Scanning electron micrograph images of wild-type (I), *cuc1 cuc2* (J), *pin1-3* (K) and *pin1-3 cuc1 cuc2* (L) embryos. Note that two splits are apparent at the top of a cup-shaped cotyledon in *cuc1 cuc2* (J, arrowheads) while no such split is found in *pin1 cuc1 cuc2* (L).

Table 1. Frequencies of cotyledon fusion phenotypes

Genotype	Frequency (%)			Total number of seedlings
	No fusion*	Partial fusion†	Cup-shaped‡	
cuc1§	99.5	0.5	0	-
cuc2§	99.5	0.5	0	-
cuc1 cuc2§	0	0	100	-
pin1-3	54.7	45.3	0	64
pin1-6	24.5	75.5	0	175
pin1-3 cuc1	0	0	100	28
pin1-6 cuc1	0	0	100	100
pin1-3 cuc2	6.1	87.9	6.1	33
pin1-6 cuc2	9.1	90.1	0.8	121
mp	45.9	54.1	0	148
mp cuc1	2.2	61.8	36	178
mp cuc2	20.5	79.5	0	156

*Cotyledons are completely separated.
†Cotyledons are partially fused but at least one of the boundaries remains separated.
‡Fused cotyledons surround the entire apex (cup-shaped type).
§Adopted from Aida et al., 1997.

embryogenesis onwards. The defect of *pin1* in cotyledon separation is much milder than that of *cuc1 cuc2*, in both the extent and frequency of fusion (Table 1). All *pin1* seedlings developed shoots, indicating that *PIN1* is not essential for SAM formation (data not shown).

To examine whether *PIN1* is functional in *cuc1 cuc2* double mutants, we constructed the corresponding triple mutants. These showed a striking phenotype, in which cotyledons were completely fused, without any trace of cotyledon boundaries (Fig. 1G). This phenotype was observed at the torpedo stage of embryogenesis (Fig. 1L). In addition, vascular bundles of cotyledons in seedlings were evenly distributed and showed a radial symmetry (Fig. 1H). Therefore, the *pin1* mutation induced a shift from bilateral to radial symmetry in the *cuc1 cuc2* background. These results suggest that *PIN1* is still active and absolutely required to establish bilateral symmetry of the apical region in the absence of *CUC1* and *CUC2* activities.

PIN1 mRNA is expressed normally in *cuc1 cuc2* embryos

To elucidate the molecular relationship between *PIN1*, *CUC1* and *CUC2*, we next questioned whether the expression of *PIN1* was affected by the *cuc1* and *cuc2* mutations. To address this question, we first analyzed the expression pattern of *PIN1* during early embryogenesis in wild type. *PIN1* mRNA was first detected in all cells during the very early stage of embryo development (data not shown), before the beginning of the expression of *CUC1* and *CUC2*. At the late globular stage (Fig. 2A), *PIN1* mRNA accumulated in the inner part of the embryo and at the future site of cotyledon emergence. During heart and torpedo stages (Fig. 2B-D), *PIN1* expression was progressively restricted to the provascular tissues both in the embryo axis and in the developing cotyledons, in a pattern very similar or identical to that of the PIN1 protein (Steinmann et al., 1999).

PIN1 mRNA expression was then analyzed in siliques of self-fertilized *cuc1/cuc1 cuc2*/+ plants, as the *cuc1 cuc2* double mutant is sterile. *PIN1* expression was identical to wild-type expression in heart- and torpedo-stage *cuc1cuc2* embryos, as revealed by the analysis of serial sections (Fig. 2E,F; data not shown). *PIN1* mRNA was detected in the provascular tissues in the embryo axis. In the cotyledons, only two provascular bundles expressing *PIN1* could be detected, confirming the existence of two morphologically distinct cotyledons in the *cuc1 cuc2* double mutant. We conclude that *PIN1* expression is not significantly affected by the *cuc1 cuc2* double mutations.

Expression of *CUC1* and *CUC2* in *pin1* embryos

Because *pin1* mutants display mild defects in cotyledon separation, a process controlled by the *CUC* genes, we questioned whether expression patterns of these genes are altered in *pin1* mutant embryos. To test this possibility, the expression patterns of *CUC1* and *CUC2* were examined at the early heart stage, shortly after the activation of their expression and the initiation of cotyledon formation. In wild type, *CUC1* and *CUC2* are expressed in a stripe between cotyledon primordia, as revealed by sagittal longitudinal serial sections (Fig. 3A-D) (Aida et al., 1999; Takada et al., 2001). The expression of each gene was detected only in the median section as a signal that extends from periphery to periphery (Fig. 3C,D; middle panels). In contrast, little or no signal was detected in the neighboring sections (Fig. 3C,D; left and right panels). At the periphery, the signal was not always restricted to the apical half of the embryo and often extended basally. Although the expression patterns of the two genes largely overlap, a slight difference between them could be observed. Within the elongated region of *CUC1* expression, the signal in the center was often weaker than in the periphery, or even undetectable (Fig. 3C, middle panel). In contrast, the *CUC2*

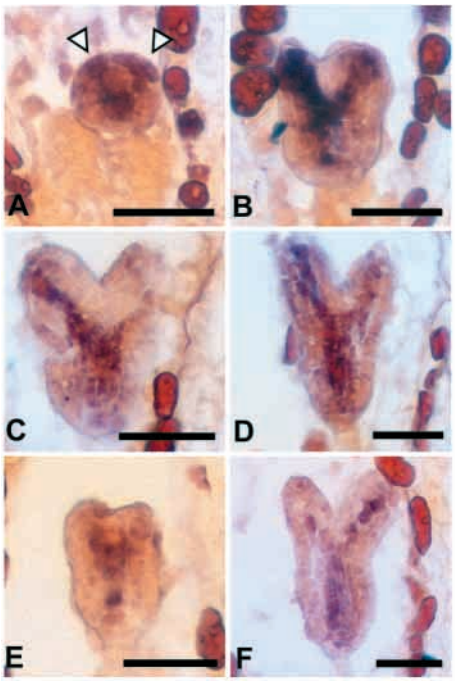

Fig. 2. Expression of *PIN1* during early embryogenesis in *cuc1 cuc2* double mutants. *PIN1* mRNA was detected using in situ hybridization. (A-D) Expression of *PIN1* in wild-type embryos. (E,F) Expression of *PIN1* in *cuc1 cuc2* embryos. Arrowheads in A indicate the future sites of cotyledon emergence. Note the similar expression pattern of *PIN1* in wild-type and *cuc1 cuc2* embryos. Scale bar, 40 μm.

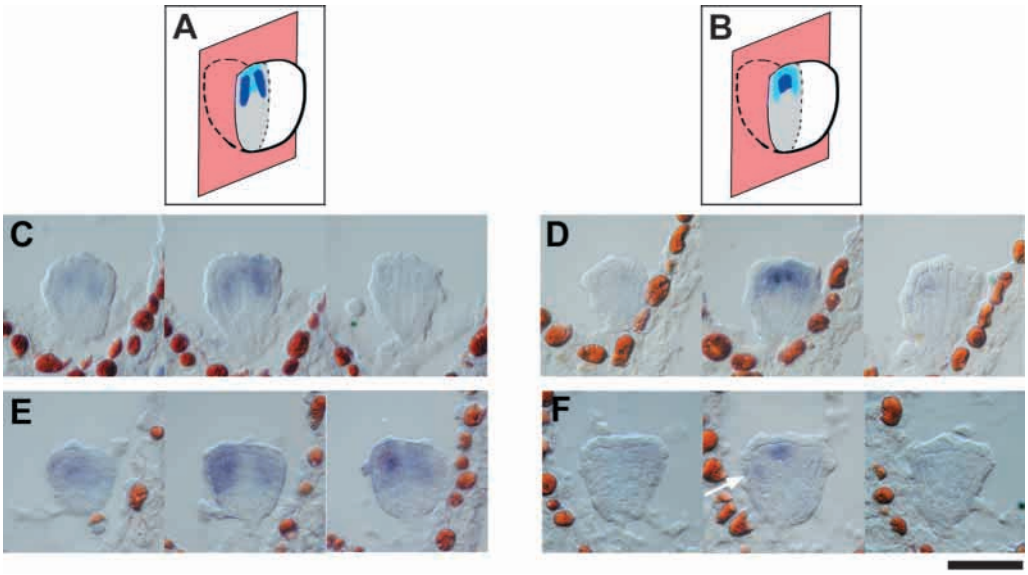

Fig. 3. Expression of *CUC1* and *CUC2* in *pin1* embryos. *CUC1* and *CUC2* mRNA was detected using in situ hybridization. (A,B) Schematic representation of wild-type expression patterns of *CUC1* (A) and *CUC2* (B) in a median sagittal section. Relative intensities of the signal are represented by dark (strong) and light (weak) blue. (C,E) *CUC1* expression in wild type (C) and *pin1-3* (E) in serial longitudinal sections. (D,F) *CUC2* expression in wild type (D) and *pin1-3* (F) in serial longitudinal sections. Arrow in F indicates an example of a weak spot of signal, which is found at the side of the embryo in a few cases. Scale bar, 50 μm for C-F.

signal was stronger in the center compared to the periphery (Fig. 3D, middle panel).

We examined the expression of *CUC1* in developing *pin1* embryos in siliques from selfed *pin1-3*/+ or *pin1*-6/+ plants, because *pin1* homozygous plants are sterile. Although, in most cases, the phenotype of *pin1* was not morphologically apparent at the early heart stage, we could find embryos with expression patterns that were not observed in wild type. In a population segregating for *pin1-3*, we found 15 out of 51 (29%) embryos with abnormal *CUC1* expression, as judged by serial sections. In most of them (13 of 15), the signal extended to a large part of the embryonic apex (Fig. 3E). The area of *CUC1* signals varied from embryo to embryo, ranging from a half to three quarters of the apex, and occasionally included bulging cotyledon primordia (Fig. 3E). In two embryos, the signal was restricted to a relatively narrow region, which occupied less than half of the apex (data not shown). Similar results were obtained when embryos segregating for *pin1-6* were examined. In this case, 8 of 41 (20%) embryos showed *CUC1* expression that expanded in a large part of the apex while the rest remains normal (data not shown). In contrast, the wild-type control did not display any of these abnormal expression patterns (n=34). These observations indicate that *CUC1* expression is variably altered and tends to expand in *pin1* embryos.

We next examined *CUC2* expression in *pin1*/+ siliques. Abnormal expression patterns were found in 12 out of 50 (24%) embryos segregating for *pin1-3* and 11 out of 47 (23%) embryos segregating for *pin1-6*. In these embryos, the signal was restricted to the center and not found in the periphery (Fig. 3F). In addition to the central signal at the apex, a weak spot of expression was found at a lateral side of the embryo axis in a few cases (Fig. 3F, arrow). In wild-type controls, we did not observe any of these abnormal patterns, except for one embryo that showed overall reduction of the signal (n=31, data not shown). These observations indicate that, in *pin1* embryos, *CUC2* expression is excluded from the periphery and confined to the center of the embryonic shoot apex.

Double mutant phenotype of *pin1 cuc1* and *pin1 cuc2*

Our expression analysis suggests that, in *pin1* mutants, *CUC2* activity may be reduced in contrast to *CUC1*. In this scenario,

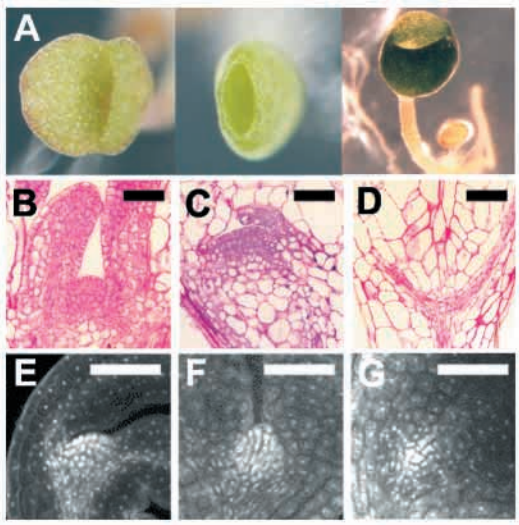

Fig. 4. Double mutant phenotypes of *pin1 cuc1*. (A, left and center) 4-day-old seedlings of *pin1-3 cuc1* with significant splits at the top (left) and no splits (center) and (right) 6-day-old seedling of *pin1-6 cuc1*. (B-D) Histological sections of the SAM stained with Toluidine Blue. (B) Wild type. (C) *pin1-6*. (D) *pin1-6 cuc1*. Scale bars, 50 μm. (E-G) Confocal images of the apices of mature embryos stained with propidium iodide. (E) Wild type. (F) *pin1-3*. (G) *pin1-3 cuc1*. The SAM is the area of small densely stained cells. Note the SAM is smaller in *pin1-3 cuc1* embryo. Scale bars, 50 μm.

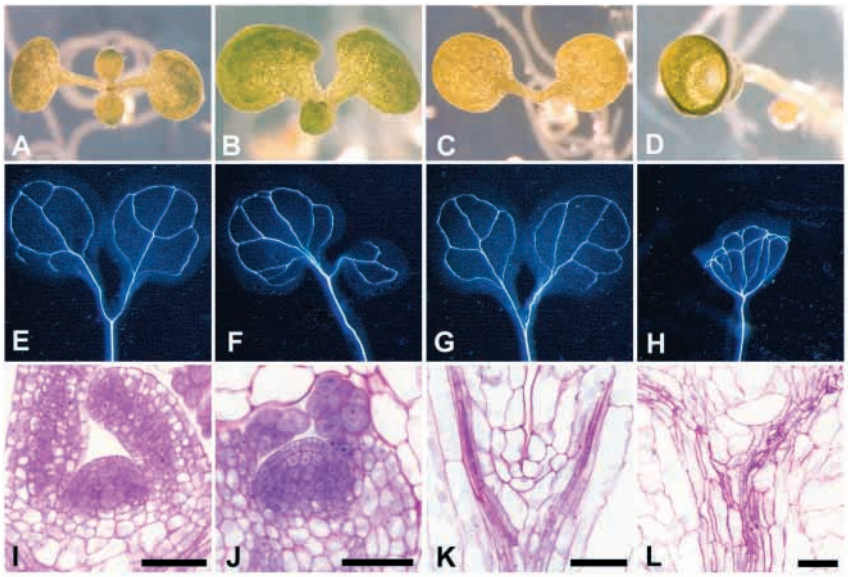

Fig. 5. Phenotype of *pin1 stm* double mutants. (A-D) 7-day-old seedlings. (A) Wild type. (B) *pin1-6* single mutant. (C) *stm* single mutant. (D) *pin1-6 stm* double mutant. (E-H) Seedlings were cleared to visualize the vascular pattern. (E) Wild type. (F) *pin1-6* single mutant. (G) *stm* single mutant. (H) *pin1-6 stm* double mutant. The vasculature shows a bilateral symmetry in wild type and *stm* while it is asymmetric in *pin1-6*. In the double mutant the vasculature shows a radial symmetry. (I-L) Histological sections of the SAM stained with Toluidine Blue. (I) Wild type. (J) *pin1-6*. (K) *stm*. (L) *pin1-6 stm*. Scale bars, 50 μm.

elimination of *CUC1* activity in a *pin1* mutant background should result in a more severe ”cup-shaped cotyledon”-like phenotype, while elimination of *CUC2* should not significantly enhance the *pin1* phenotype. To test this model, we constructed *pin1 cuc1* and *pin1 cuc2* double mutants to genetically eliminate *CUC1* and *CUC2* activities from the *pin1* mutant background, respectively.

cuc1 enhanced the *pin1* phenotype in both the extent and frequency of fusion. The double mutants had fused cotyledons, forming a cup-shaped structure that surrounded the entire seedling apex (Fig. 4A; Table 1). However, the extent of fusion varied among seedlings. Some had splits at the top (Fig. 4A, left panel) while the others showed complete fusion (Fig. 4A, middle and right panels), similar to those in *pin1 cuc1 cuc2* triple mutants. The vascular pattern of the double mutant seedlings was significantly disturbed and did not show bilaterally symmetrical patterns (data not shown).

In addition to the deficiencies in cotyledon separation and bilateral symmetry, the *pin1 cuc1* double mutants were defective in SAM formation. This phenotype was most prominent in *pin1-6 cuc1* double mutants where the primary SAM was completely absent, in contrast to wild type, *pin1-6* and *cuc1* single mutants (Fig. 4B-D, data not shown for *cuc1*). Interestingly, the *pin1-6 cuc1* double mutant seedlings could develop adventitious SAMs from the base of fused cotyledons several days after germination (data not shown). In contrast, some *pin1-3 cuc1* mutants developed a primary SAM, although often reduced in size compared to wild type and *pin1-3* single mutants (Fig. 4E-G). The difference between *pin1-6 cuc1* and *pin1-3 cuc1* phenotypes may reflect a difference in the strength of the *pin1* alleles.

The phenotype of *pin1 cuc2* was intermediate between those of *pin1* and *cuc1 pin1*. In most cases, the abnormal seedlings of *pin1 cuc2* were morphologically indistinguishable from those of *pin1* single mutants (data not shown). However, fusion of cotyledons occurred more frequently than in *pin1* single mutants (Table 1). Seedlings with a ‘cup-shaped cotyledon’, in which fused cotyledons surrounded the entire apex, were observed only in a few cases (Table 1).

Taken together, our results show that the *cuc1* mutation greatly enhances the *pin1* phenotype while *cuc2* only moderately does so, suggesting that *CUC1* still shows significant activity while *CUC2* activity is much reduced in the *pin1* mutant. These results thus provide functional support for the indications from our expression analysis of *CUC1* and *CUC2* in *pin1* embryos.

Phenotype of the *pin1 stm* double mutant

We have previously shown that *CUC1* and *CUC2* are essential for the activation of *STM* and that *STM* may cooperate with

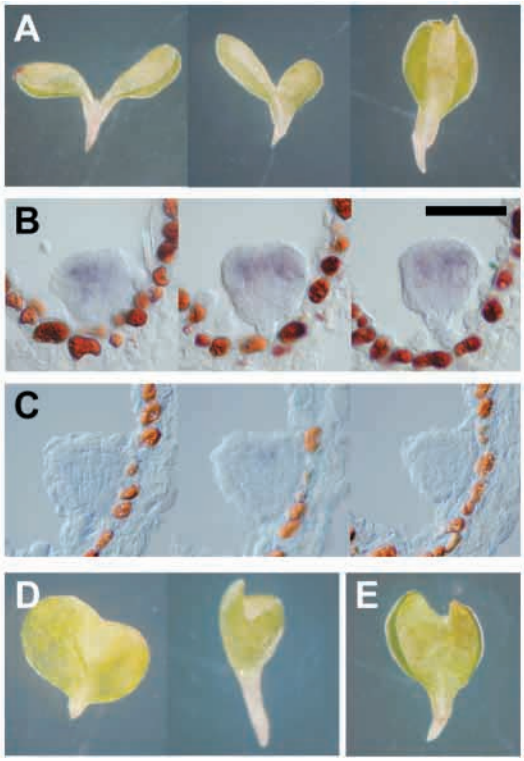

Fig. 6. Relationship between *CUC* and *MP* genes. (A) *mp* seedlings with completely separated (left) and variably fused (middle and right) cotyledons. Note that the size of the cotyledons is unequal in the middle seedling. (B,C) Expression patterns of *CUC1* (B) and *CUC2* (C) in serial longitudinal sections of *mp* embryos. *CUC1* and *CUC2* mRNA was detected using in situ hybridization. Scale bar, 50 μm for B and C. (D) *mp cuc1* seedlings. The seedling at the left has cotyledons fused at one side while the seedling at the right has a ‘cup-shaped’ cotyledon. (E) *mp cuc2* seedling with cotyledons fused at one side.

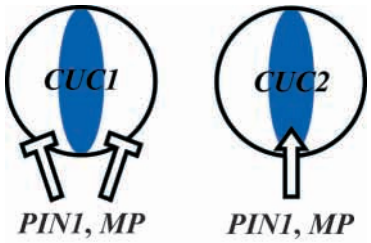

Fig. 7. Regulation of expression patterns of *CUC1* and *CUC2* at the early heart stage. *PIN1* and *MP* repress *CUC1* expression in the cotyledons and promote *CUC2* in the cotyledon boundaries.

CUC1 and *CUC2* in cotyledon separation (Aida et al., 1999; Takada et al., 2001). Conversely, *STM* is required for proper spatial expression of *CUC1* and *CUC2* during late embryogenesis. In order to complete our understanding of the role of *PIN1* in the genetic pathway controlling cotyledon separation and meristem initiation, we analyzed the *pin1 stm* double mutant. *STM* and *PIN1* are both located on chromosome 1, respectively at positions 75 and 103 cM on the classical genetic map (TAIR resource, http://www.arabidopsis.org). *pin1 stm* double mutants were recognized as seedlings, the frequency of which was only 3.2% (*n*=398), which can be explained by the genetic linkage between the two genes.

pin1 stm double mutants showed a complete fusion of the cotyledons (Fig. 5D), exhibiting a phenotype very similar to that of the *pin1 cuc1 cuc2* triple mutants. This phenotype was never observed in single mutants of *pin1* or *stm* (*n*=285 and *n*=265 for homozygous mutants, respectively). The vascular bundles were radially distributed in the *pin1 stm* double mutant seedlings, in contrast to wild-type and *stm* single mutant seedlings, which exhibited a bilateral symmetry (Fig. 5E-H). In the *stm* single mutant, fusion sometimes occurred on one side at the base of the cotyledon, resulting in a small change in the angle formed by the two cotyledons (data not shown). However, these seedlings still retained bilateral symmetry. Thus, the *pin1* mutation changes the symmetry of the seedlings from bilateral to radial in the *stm* mutant background, as it does in the *cuc1 cuc2* background. As in the *stm* single mutant, *pin1 stm* did not form a SAM, showing that the defect of *stm* in SAM formation was not affected by the presence of *pin1* mutation (Fig. 5I-L).

Thus, like in the *pin1 cuc1 cuc2* triple mutant, cotyledon separation and establishment of bilateral symmetry do not occur in the *pin1 stm* background. This result further confirms the importance of *STM* in promoting cotyledon separation during embryogenesis.

The *mp* mutation affects *CUC1* and *CUC2* activity in a similar way to *pin1*

The *mp* mutant, which lacks a hypocotyl and a root, often shows cotyledon defects similar to *pin1*, including fusion and reduction in size (Berleth and Jürgens, 1993) (Fig. 6A; Table 1). Fusion always occurs on one side so that fused cotyledons never surrounded the entire axis (Fig. 6A, middle and right panels). Cotyledons are often asymmetric and of a different size (Fig. 6A, middle panel). In contrast to *pin1*, however, an increase in the number of cotyledons is rarely observed (data not shown). These observations demonstrate that the *mp* mutation affects bilateral symmetry and cotyledon separation. This prompted us to investigate the relation between *MP*, *CUC1* and *CUC2*.

We first examined the expression of the *CUC* genes in *mp* mutant embryos. In 13 *mp* embryos at the early heart stage, 10 showed expanded expression of *CUC1* at the periphery of the apex as observed in *pin1* (Fig. 6B). The area of *CUC1* expression often included the outgrowing cotyledon primordia. In contrast, *CUC2* expression was reduced and confined to the center. *CUC2* was not observed in the periphery in 8 out of 12 *mp* embryos (Fig. 6C). As observed in *pin1*, a weak spot of signal was observed on a lateral side of the embryo axis in a few cases (data not shown). These results showed that, during cotyledon initiation, *CUC1* expression was maintained or even expanded in the embryonic shoot apex while *CUC2* expression was reduced in *mp* embryos.

We next examined the double mutants of *mp cuc1* and *mp cuc2*. In *mp cuc1*, cotyledon fusion occurred much more frequently than in *mp* (Table 1). In addition, a significant number of seedlings showed the cup-shaped fusion phenotype, in which cotyledon surrounded the entire apex (Fig. 6D). The frequency of this type of fusion, however, was not as high as in *pin1 cuc1* (Table 1). In contrast, the *mp cuc2* double mutant showed a phenotype intermediate between those of *mp* and *mp cuc1*. Cotyledon fusion occurred at a higher frequency than in *mp* and at a lower frequency than in *mp cuc1* (Table 1). The seedlings of *mp cuc2* that displayed cotyledon fusions were morphologically indistinguishable from *mp* single mutants with fused cotyledons while cup-shaped seedlings were not observed (Fig. 6E). These results suggest that *CUC1* activity is maintained while *CUC2* activity is reduced in *mp*. Therefore, the expression and double mutant analyses indicate that the *mp* mutation affects *CUC1* and *CUC2* activities in a way similar to *pin1*.

DISCUSSION

PIN1, *MP*, *CUC1*, *CUC2* and *STM* are part of the network of genes controlling apical patterning in the embryo

In wild-type embryos, the *CUC* genes are both expressed in a stripe between the two cotyledon primordia at early heart stage. In *pin1* and *mp* embryos, *CUC1* expression tends to expand into the periphery of the apex while *CUC2* expression is reduced and confined to a small spot at the center. *PIN1* and *MP* initiate their expression from the very early stages of embryogenesis, prior to *CUC* gene expression (Hardtke and Berleth, 1998; Steinmann et al., 1999) (this analysis). Consistent with the effect of *PIN1* and *MP* on *CUC* gene expression, the *cuc1* mutation greatly enhanced the cotyledon fusion phenotypes of *pin1* and *mp*, suggesting that *CUC1* activity remains at a significant level and is largely responsible for cotyledon separation in these mutant backgrounds. In contrast, *cuc2* only moderately enhanced the *pin1* and *mp* phenotypes, suggesting that *CUC2* activity is significantly reduced in these mutants. Together, these data suggest two roles for *PIN1* and *MP* in the spatial regulation of *CUC* gene expression (Fig. 7): (1) *PIN1* and *MP* are required for repression of *CUC1* in the cotyledons; (2) *PIN1* and *MP* are

required for activation of *CUC2* in the cotyledon boundaries. We propose that *PIN1* and *MP* are important factors involved in setting up the bilateral pattern of *CUC* gene expression.

Previous analyses have shown that *CUC1* and *CUC2* are redundantly required for the activation of *STM*, which in turn helps to maintain the spatial expression pattern of the *CUC* genes during later stages of embryogenesis (Aida et al., 1999; Takada et al., 2001). In accordance with this, the *stm* mutant shows some cotyledon fusion, suggesting a partial loss of *CUC* function. In this analysis, we found that the *stm* mutation strongly enhances the *pin1* phenotype and actually mimics the *pin1 cuc1 cuc2* phenotype. Considering that the *pin1* single mutation strongly reduces *CUC2* activity and has only a limited effect on *CUC1*, this result suggests that *STM* is required for maintaining *CUC1* activity in the *pin1* mutant background. Interestingly, cotyledon fusion in *pin1 stm* is complete, indicating that the defect already occurs at a very early stage of cotyledon development. This raises the possibility that the effect of *STM* on *CUC1* activity starts much earlier. Given that the activity of *CUC2* is greatly reduced in the *pin1* single mutant background, the *pin1 stm* double mutant phenotype does not give any genetic evidence for a similar effect of *STM* on early *CUC2* expression. However, this possibility cannot be discarded.

It is important to stress that *CUC1* and *CUC2* are homologous and have highly redundant functions as indicated by the very subtle phenotypes of the single mutants and similar but not identical expression patterns (Aida et al., 1997; Ishida et al., 2000; Takada et al., 2001). However, their behavior in the *pin1* and *mp* backgrounds shows that they are differently regulated during embryogenesis. The biological significance of this observation is not yet clear. Differential regulation in duplicated gene pairs is found in a number of species and may play a role in the evolution of functional divergence (Force et al., 1999; Pickett and Meeks-Wagner, 1995). In addition, the differential regulation of functionally redundant factors might provide developmental stability and buffer possible physiological or genetic perturbations.

How do the *PIN1* and *MP* genes regulate the expression of the *CUC* genes?

The *PIN1* gene encodes a transmembrane protein, which is thought to act as a catalytic auxin efflux carrier (Gälweiler et al., 1998; Palme and Gälweiler, 1999). In accordance with this, polar auxin transport is severely reduced in the mutant (Okada et al., 1991). In addition, micro-application of exogenous auxin on the inflorescence meristem can rescue the defects of *pin1* in organ formation in a position-dependent manner (Reinhardt et al., 2000). These results indicate that the mutation disrupts the spatial distribution of auxin, which causes the observed phenotype. *MP* encodes a member of the ARF family of transcription factors, which bind to auxin responsive elements in the promoters of auxin-regulated genes (Hardtke and Berleth, 1998; Ulmasov et al., 1997). Some of the defects of *mp* are found in other auxin response mutants, such as *bodenlos*, *auxin-resistant6* and *iaa18* (Hamann et al., 1999; Hobbie et al., 2000; Reed, 2001). These results suggest that *MP* is involved in mediating auxin signals and thus functions downstream of *PIN1* and auxin transport. Another possibility, not mutually exclusive to the former, is that *MP* could affect auxin transport itself. This could either be due to a direct influence of *MP* on auxin transporters or an indirect effect, due to the lack of vascular continuity, for instance, which might be important for efficient auxin transport (Przemeck et al., 1996).

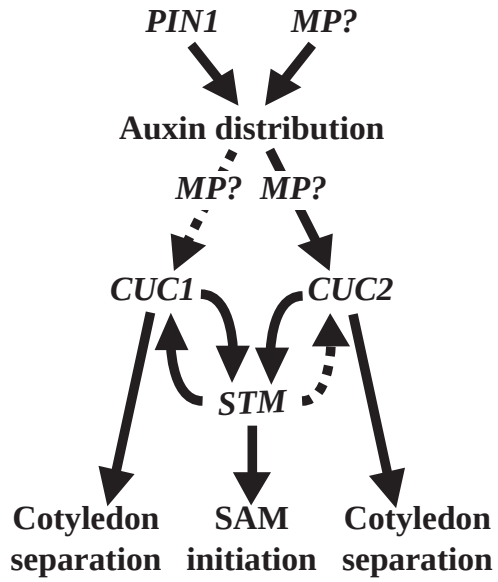

Fig. 8. A model for the patterning of the apical region of the embryo. Solid arrows indicate activation. Dotted arrows indicate an effect on spatial expression. *PIN1* promotes auxin transport and creates a specific auxin distribution. The auxin distribution activates the expression of *CUC2* and influences the spatial expression pattern of *CUC1*. *MP* regulates *CUC* gene expression in response to auxin signals. *MP* could also contribute to auxin distribution through promoting auxin transport. *CUC1* and *CUC2* redundantly promote cotyledon separation as well as expression of *STM*, the latter of which in turn promote SAM formation. *STM* also contributes to cotyledon separation through regulating spatial expression of *CUC1* and *CUC2* at the late embryo. In addition, *STM* promotes the *CUC1* activity from early embryogenesis on.

Given the data on *PIN1* and *MP* function, how could these genes affect *CUC* gene expression? Immunolocalization studies (Steinmann et al., 1999) suggest that *PIN1*-dependent auxin transport may already be active in the globular embryo before *CUC* expression is initiated. Likewise, *MP* is expressed from about the same stage onwards (Hardtke and Berleth, 1998). Combined with our data, this would suggest a scenario where *PIN1* and *MP*, via their control on auxin fluxes and responses, regulate the expression of *CUC1* and *CUC2*. It remains to be established whether *MP* functions downstream of *PIN1*, i.e. by regulating the expression of *CUC1* and *CUC2* in response to the auxin distribution established by *PIN1*. At this stage, we do not know if the *CUC* genes are directly regulated by auxin or if the regulation of their expression is a secondary consequence of changes in cell identity induced by auxin. It is noteworthy that *MP* is expressed in all subepidermal cells, including most of the *CUC1* and *CUC2* expression domain at the globular stage (Hardtke and Berleth, 1998). Recently, the *NAC1* gene, another member of the NAC family, was implicated in auxin signal transduction in the root (Xie et al., 2000). Application of auxin induces *NAC1* in a way similar to early auxin-responsive genes. In this context, it is interesting to note that the *CUC2* gene contains a potential auxin responsive sequence (M. A. and M. T., unpublished results).

Although the significance of this sequence is not known, *CUC2* may be able to respond to auxin.

An effect of *PIN1* on *CUC2* expression in the inflorescence meristem was reported previously (Vernoux et al., 2000). In this case, however, *CUC2* expression was expanded in the *pin1* mutant, suggesting that *PIN1* limits *CUC2* expression at the inflorescence SAM. This might seem contradictory to our finding that *PIN1* positively influences *CUC2* expression in the embryo, but the data might simply suggest that auxin distributions caused by *PIN1* are different in the apex of embryos and inflorescences. Low concentrations of auxin, for example, might stimulate *CUC2* expression, whereas high concentrations might inhibit the same gene. Alternatively, *CUC2* may react to auxin differently in each tissue. To understand exactly how the distribution of the hormone evolves during the plant life cycle, we need to develop systems to monitor auxin concentrations at the cellular level in the shoot apex. Nevertheless, the *pin1* phenotype and its effect on *CUC2* expression clearly illustrate that the situation in the inflorescence meristem is different from the one at the embryonic apex.

Establishment of bilateral symmetry is dependent on *PIN1* and *MP* activities

With the outgrowth of the cotyledons, the symmetry of the embryo apex changes from radial to bilateral. The *pin1* and *mp* mutations disrupt this change, as was reflected by the random positioning, partial fusion and asymmetric outgrowth of cotyledon primordia and disorganized expression patterns of the *CUC* genes. These results further strengthen the idea that auxin plays an important role in establishment of bilateral symmetry during embryogenesis, as previously suggested by physiological studies in the *Brassica juncea* embryo (Hadfi et al., 1998; Liu et al., 1993).

The effect of *pin1* on bilateral symmetry is even more striking in the *cuc1 cuc2* and *stm* backgrounds. When these mutations are combined with *pin1*, the well-defined bilateral symmetry of the cotyledons, with two major vascular bundles, becomes a fully radially symmetrical wine glass shaped cotyledon, with multiple vascular bundles distributed evenly. Thus, in *pin1 cuc1 cuc2* and *pin1 stm*, the symmetry transition during embryogenesis appears completely inhibited and the radial symmetry of the globular embryo is retained. The expression pattern of *PIN1* in the embryo is consistent with its key function in this transition of symmetry. In the late globular embryo, *PIN1* mRNA accumulates in two symmetrical groups of cells that will give rise to the cotyledons. Therefore, *PIN1* could control the positioning of cotyledon primordia in a similar way to that proposed for the initiation of floral primordia (Vernoux et al., 2000). One simple model is that an auxin distribution with a bilaterally symmetric pattern is formed by *PIN1* and subsequently initiates the morphological changes associated with symmetry transition. *MP* could also be involved in this process through perception of the distribution of auxin. Alternatively, as previously discussed, *MP* could be involved in formation and/or maintenance of auxin distribution.

The expression pattern of *PIN1* is initially ubiquitous and then becomes bilaterally symmetric by late globular stage. How this expression pattern is regulated is unknown. Since the *PIN1* activity is required for the transition of symmetry in the early embryo, one possibility is that *PIN1* itself is involved in the establishment of the bilateral pattern of *PIN1* expression. Examination of *PIN1* expression in non-null *pin1* mutant backgrounds is required to test this possibility. Alternatively, the bilateral expression pattern of *PIN1* might be regulated by other factors. The *cuc1 cuc2* double mutations do not markedly affect *PIN1* expression, demonstrating that at least *CUC1* and *CUC2* are not essential for this regulation.

Both the *cuc1 cuc2* double mutations and the *stm* single mutation synergistically enhance the dissymmetric phenotype of *pin1*, raising the possibility that *CUC1*, *CUC2* and *STM* also have roles in establishing bilateral symmetry. Moreover, the expression patterns of these genes show bilateral symmetry during late globular stage, which could be essential for the symmetry change. However, a role of these genes in establishing bilateral symmetry is in contradiction with the phenotypes of the mutants: *cuc1 cuc2* double mutants can still establish bilateral symmetry. In the single mutants of *cuc1*, *cuc2* and *stm*, a fraction of seedlings shows cotyledon fusion on one side. In all cases, the relative position of the cotyledons shifts towards the fused part, but bilateral symmetry is retained. The size of cotyledons is not altered and the seedlings still have a plane of symmetry (M. A. and M. T., unpublished observation). These observations suggest that reduced activity in cotyledon separation perturbs cotyledon positioning, but not the symmetry transition per se. Together, the data suggest a scenario where *PIN1* and *MP* initiate the symmetry transition and cotyledon separation. In contrast, *CUC1*, *CUC2* and *STM* are not essential for the symmetry transition and may indirectly contribute to the stabilization of the symmetry through maintaining proper cotyledon position. Analysis of the relationships between *PIN1*, *MP* and other genes involved in patterning of the embryonic apex, such as *PINOID*, *AINTEGUMENTA* and *ASYMMETRIC LEAVES1* (Bennett et al., 1995; Byrne et al., 2000; Long and Barton, 1998) will be important for a better understanding of the processes regulating embryo symmetry.

Conclusions

Our results allow us to present a model for the patterning of the apical part of the embryo (Fig. 8). *PIN1*, possibly by regulating polar auxin transport, activates the expression of *CUC2* and is also necessary for the proper spatial expression of *CUC1*. *MP* acts similarly, either by modulating the sensitivity to auxin, or by promoting auxin transport. *CUC1* and *CUC2* activate the expression of *STM*, which, in turn, is necessary for *CUC1* activity during early embryogenesis and for *CUC2* spatial expression during later stages. The activities of *CUC1* and *CUC2* have a major role in setting up the boundaries of the cotyledon while *STM* activity is mainly responsible for the formation of the SAM. The roles of *PIN1* and *MP* in promoting primordia formation and establishment of bilateral symmetry are not included in the model.

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