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.”
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 embryonic, 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 (Ler) and Wassilewskija (WS) were used in this study. The following mutant alleles were used: cuc1-1 (Ler) (Takada et al., 2001), cuc2 (Ler) (Aida et al., 1997), pin1-3 (Ler) (Bennett et al., 1995), pin1-6 (WS) (Vernoux et al., 2000), stm-dgh6 (WS) and mp-rtl (Ler). 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 (Bechthold 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 alleleism 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. F2 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 F3 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 plants homozygous for cuc1-1 or cuc2. Among F2 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 F3 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 heterozygous were made. Double mutants could be recognized in the segregating F2 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 NaH2PO4, 3 mM NaHPO4) 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
embryogenesis onwards. The defect of \textit{pin1} in cotyledon separation is much milder than that of \textit{cuc1 cuc2}, in both the extent and frequency of fusion (Table 1). All \textit{pin1} seedlings developed shoots, indicating that \textit{PIN1} is not essential for SAM formation (data not shown).

To examine whether \textit{PIN1} is functional in \textit{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 \textit{pin1} mutation induced a shift from bilateral to radial symmetry in the \textit{cuc1 cuc2} background. These results suggest that \textit{PIN1} is still active and absolutely required to establish bilateral symmetry of the apical region in the absence of \textit{CUC1} and \textit{CUC2} activities.

\textit{PIN1} mRNA is expressed normally in \textit{cuc1 cuc2} embryos

To elucidate the molecular relationship between \textit{PIN1}, \textit{CUC1} and \textit{CUC2}, we next questioned whether the expression of \textit{PIN1} was affected by the \textit{cuc1} and \textit{cuc2} mutations. To address this question, we first analyzed the expression pattern of \textit{PIN1} during early embryogenesis in wild type. \textit{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 \textit{CUC1} and \textit{CUC2}. At the late globular stage (Fig. 2A), \textit{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), \textit{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 \textit{PIN1} protein (Steinmann et al., 1999).

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

Expression of \textit{CUC1} and \textit{CUC2} in \textit{pin1} embryos

Because \textit{pin1} mutants display mild defects in cotyledon separation, a process controlled by the \textit{CUC} genes, we questioned whether expression patterns of these genes are altered in \textit{pin1} mutant embryos. To test this possibility, the expression patterns of \textit{CUC1} and \textit{CUC2} were examined at the early heart stage, shortly after the activation of their expression and the initiation of cotyledon formation. In wild type, \textit{CUC1} and \textit{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 \textit{CUC1} expression, the signal in the center was often weaker than in the periphery, or even undetectable (Fig. 3C, middle panel). In contrast, the \textit{CUC2}
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,
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 

cuc1 

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...
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 pin1 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).

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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