Arabidopsis CUP-SHAPED COTYLEDON3 Regulates Postembryonic Shoot Meristem and Organ Boundary Formation

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Overall shoot architecture in higher plants is highly dependent on the activity of embryonic and axillary shoot meristems, which are produced from the basal adaxial boundaries of cotyledons and leaves, respectively. In Arabidopsis thaliana, redundant functions of the CUP-SHAPED COTYLEDON genes CUC1, CUC2, and CUC3 regulate embryonic shoot meristem formation and cotyledon boundary specification. Their functional importance and relationship in postembryonic development, however, is poorly understood. Here, we performed extensive analyses of the embryonic and postembryonic functions of the three CUC genes using multiple combinations of newly isolated mutant alleles. We found significant roles of CUC2 and CUC3, but not CUC1, in axillary meristem formation and boundary specification of various postembryonic shoot organs, such as leaves, stems, and pedicels. In embryogenesis, all three genes make significant contributions, although CUC3 appears to possess, at least partially, a distinct function from that of CUC1 and CUC2. The function of CUC3 and CUC2 overlaps that of LATERAL SUPPRESSOR, which was previously shown to be required for axillary meristem formation. Our results reveal that redundant but partially distinct functions of CUC1, CUC2, and CUC3 are responsible for shoot organ boundary and meristem formation throughout the life cycle in Arabidopsis.

INTRODUCTION

In higher plants, production of most shoot organs is dependent on the activity of small tissue called the shoot meristem, which maintains pluripotent stem cells at its tip. In the normal life cycle of the plant, formation of shoot meristems occurs during both embryogenesis and postembryonic development. The embryonic shoot meristem is initiated at the axil of cotyledon(s), the first leaf-like lateral organ produced from the flank of the embryo apex, and is an ultimate source of nearly all shoot organs produced during subsequent postembryonic development (Aida and Tasaka, 2001). In contrast with embryonic meristem formation that occurs only once, formation of postembryonic shoot meristems can occur multiple times at multiple locations. These postembryonic meristems are generally initiated from the axil of leaves and thus are referred to as axillary shoot meristems. Axillary meristems are initiated and develop to form branches in response to environmental and developmental cues, elaborating the whole architecture of adult plants (McSteen and Leyser, 2005).

A number of genes essential for embryonic shoot meristem formation have been identified (Barton and Poethig, 1993; Lauk et al., 1996; Souer et al., 1996; Emery et al., 2003; Weir et al., 2004). Arabidopsis thaliana CUP-SHAPED COTYLEDON genes CUC1 and CUC2 (Aida et al., 1997; Takada et al., 2001) encode closely related members of the NAC transcription factor family. Due to the redundant functions of these genes, neither the cuc1 nor cuc2 single mutation results in severe seedling phenotype, but the cuc1 cuc2 double mutation causes a complete lack of embryonic shoot meristem formation (Aida et al., 1997). Besides their roles in shoot meristem initiation, CUC1 and CUC2 are also involved in specification of shoot organ boundaries. Expression of CUC1 and CUC2 is detected in boundaries of various shoot organ primordia, including those of cotyledons, and the double mutation in CUC1 and CUC2 causes ectopic tissue growth at the cotyledon boundaries, resulting in fusion of the organs (Aida et al., 1997, 1999; Ishida et al., 2000; Takada et al., 2001). Another Arabidopsis CUC gene, CUC3, encoding a NAC protein of a different clade from the one for CUC1, CUC2, and their putative orthologs in other species (Zimmermann and Werr, 2005), also participates in embryonic shoot meristem formation and cotyledon boundary formation redundantly with CUC1 and CUC2 (Vroemen et al., 2003). These results indicate a close relationship between embryonic shoot meristem formation and specification of cotyledon boundaries (Aida and Tasaka, 2006a, 2006b).

A number of mutants show frequent loss of axillary shoot meristem formation, and some of them also indicate a link between the boundary of postembryonic shoot organs and axillary meristem formation (Schmitz and Theres, 2005). Mutations in the LATERAL SUPPRESSOR (LAS) gene of Arabidopsis result in frequent lack of axillary shoot meristem formation at the rosette leaf axils (Greb et al., 2003). Expression of LAS is
detected in a region along the boundary between the shoot meristem and initiating leaf primordia. This expression pattern continues in the basal boundary of the leaf primordia throughout their development, and a subpopulation of the LAS-expressing cells eventually form the axillary meristem. The MYB encoding gene REGULATOR OF AXILLARY MERISTEMS1 (RAX1) of Arabidopsis promotes axillary meristem initiation together with its redundant homologs RAX2 and RAX3 (Keller et al., 2006; Müller et al., 2006). Expression of RAX1 is initially detected in a subregion along the boundary between the meristem and initiating leaf primordia.

In Antirrhinum majus, single mutations in the CUPULIFORMIS gene, an ortholog of CUC1 and CUC2 of Arabidopsis, results in striking fusion phenotypes in nearly all shoot organs, including cotyledons, leaves, stems, floral organs, and ovules, indicating a role for this gene in a wide range of shoot organ boundaries (Weir et al., 2004). Moreover, the cup mutant frequently lacks branch formation from the leaf axils, raising a possibility that the mutation also compromises axillary meristem initiation. By contrast, the cuc1 cuc2 double mutation in Arabidopsis only causes fusion of some floral organs and does not markedly alter overall architecture of the plant during postembryonic development (Aida et al., 1997). The striking difference in the postembryonic phenotypes between cup and cuc1 cuc2 raises the possibility that the Arabidopsis genome has other factor(s) that act redundantly with CUC1 or CUC2 in boundary specification and axillary shoot meristem formation during postembryonic development. CUC3 may represent a candidate for such a gene, but its role and genetic relationship with CUC1 and CUC2 in postembryonic development remain unknown.

Here, we report identification of several strong mutant alleles of cuc3 from a phenotypic enhancer mutant screening of cuc2. Extensive phenotypic analysis of multiple mutant combinations of cuc1, cuc2, and cuc3 indicates that CUC2 and CUC3 are mainly required for axillary meristem initiation and boundary specification in various postembryonic organs, including stems, pedicels, and leaves. On the other hand, all three CUC genes have significant contributions for embryonic shoot meristem and cotyledon boundary formation, and it appears that the extent of functional redundancy between CUC1 and CUC2 is greater than that between CUC3 and CUC1 or between CUC3 and CUC2. Moreover, the function of CUC3 is partially overlapping with the LAS gene in axillary meristem formation and specification of boundaries between the stem and pedicels and between cotyledons.

RESULTS

Isolation of cuc2 Enhancer Mutants

Seedlings of the cuc2 single mutant show essentially no obvious phenotype except for a very small fraction with fused cotyledons along one side (Figure 1A; Aida et al., 1997). To identify genes that function redundantly with CUC2, a 3500 M2 population of ethyl methanesulfonate (EMS)-mutagenized cuc2-1 plants was screened for strong fused cotyledon phenotype at the seedling stage.

Figure 1. Seedling Phenotypes of cuc2 Enhancer Mutants. (A) cuc2-1 single mutant. (B) to (H) cuc2 enhancer mutants. Representative seedlings from the four phenotypic categories (see text). The first class, V63 (B); the second class, X84 ([C] to [E]) and U124 (F); the third class, H48 (G); and the fourth class, T123 (H). (C) and (D) represent two typical variations from the second class, namely, cup-shaped with strong cotyledon fusion (C) and stm-like with mild fusion at the base of cotyledons (D). Arrow in (E) indicates a shoot produced from the base of the cotyledon.

Fourteen independent lines were isolated and categorized into four classes according to their phenotypes (Figures 1B to 1H, Table 1). In the first class (six lines), all seedlings completely lacked an embryonic meristem, resembling the cuc1-1 cuc2-1 double mutant (Figure 1B; Aida et al., 1997). Accordingly, all these lines contained point mutations in the CUC1 coding sequence, representing new strong alleles of cuc1 (Table 1; see Supplemental Figure 1A online). Seedlings of the second class either exhibited cup-shaped fusions (Figure 1C) or partial fusions along both edges of the cotyledons at the base (Figures 1D and 1F). The latter phenotype was similar to that observed in the stm mutant (Endrizzi et al., 1996) and thus was referred to as stm-like cotyledons. Both types of seedlings occasionally initiated a shoot from the base of cotyledons 1 to 2 weeks after germination (Figure 1E) and developed fertile plants. One line of this class, U124, contained a point mutation in CUC1, representing a weak allele of cuc1 and was designated cuc1-6 (Figure 1F, Table 1; see Supplemental Figure 1B online). The predicted mutant protein of cuc1-6 contains an amino acid substitution in the C-terminal W-motif, which is required for transactivation activity (Taoka et al., 2004). The other three lines of the second class (X84, B24, and E68) represented novel alleles of CUC3 and were subjected to further analysis (see below).

Seedlings of the third class (three lines) displayed a short root phenotype in addition to the occurrence of cup-shaped cotyledons at a low frequency (data not shown; Figure 1G). All these lines carried mutations in the gene encoding At BRM, a putative chromatin remodeling ATPase (Farrona et al., 2004; Kwon et al., 2006). In the fourth class (one line), seedlings displayed stunted cotyledons with occasional cup-shaped fusion (Figure 1H) and deformed vasculature (data not shown). This mutant contained a point mutation in the GNOM gene encoding an ARF-GEF, which was required for proper localization of the auxin efflux protein PIN1 (Steinmann et al., 1999). Isolation of atbrm and gnom
mutant alleles as phenotypic enhancers of cuc2 is consistent with the importance of chromatin remodeling and auxin transport, respectively, in the regulation of CUC gene expression (Aida et al., 2002; Furutani et al., 2004; Kwon et al., 2005, 2006).

Identification of Strong cuc3 Mutant Alleles as cuc2 Phenotypic Enhancers

The mutation in the line X84 of the second class was mapped between ATPASE and nga982 on chromosome 1. This region contains the CUC3/At1g76420 gene encoding a NAC domain protein related to CUC1 and CUC2 (Vroemen et al., 2003). Three lines of the second class identified from the initial enhancer screen (X84, B24, and E68) carried mutations in the CUC3 locus and thus were designated cuc3-101, cuc3-102, and cuc3-103 (Table 1; see Supplemental Figures 1A and 1C online). In addition, another enhancer mutant allele, cuc3-104, was independently recovered from an EMS-mutagenized population of cuc2-1 (Table 1; see Supplemental Figure 1A online). We also obtained T-DNA or transposon insertion mutants of CUC1 (cuc1-1 and cuc1-13), CUC2 (cuc2-2 and cuc2-3), and CUC3 (cuc3-105) (see Supplemental Figure 1C online; see Methods). The double mutant of cuc2-3 cuc3-105 exhibited the cup-shaped cotyledon and occasionally developed fertile shoots, similar to cuc2-1 cuc3-101, whereas each single mutant displayed weak cotyledon fusion at very low frequencies (Table 2). To confirm that mutations in CUC3 act as enhancers of the cuc2 phenotype, we transformed homozygous cuc3-2 cuc3-105 plants with a 6.1-kb genomic fragment that includes the coding sequence of CUC3. The obtained transgenic lines showed normal seedling appearance similar to the cuc2 single mutant (see Methods).

The cuc3-101 allele contained a single nucleotide substitution in the junction of the first exon and intron (see Supplemental Figure 1C online). RT-PCR analysis showed that the mutation caused altered size and reduced level of CUC3 transcripts (data not shown). cuc3-102, cuc3-103, and cuc-104 caused substitutions of conserved amino acids in the NAC domain (see Supplemental Figure 1A online).

Vroemen et al. (2003) reported a hypomorphic mutant allele of CUC3 (cuc3-1), which slightly enhances the cuc2-1 single mutant phenotype. In cuc2-1 cuc3-1, the frequency of seedlings with a cup-shaped cotyledon was only 0.2% (Vroemen et al., 2003). By contrast, >79% of seedlings had cup-shaped cotyledons in cuc2-1 cuc3-101 or cuc2-3 cuc3-105 (Table 2). The T-DNA insertion site of cuc3-105 approximately corresponds to the middle of the NAC domain–encoding sequence, suggesting that the insertion causes deletion of a large portion of the protein. We thus conclude that both cuc3-101 and cuc3-105 represent strong alleles and possibly nulls. We performed further characterization of CUC3 function using cuc3-101 and cuc3-105 as representative alleles.

Genetic Interactions among the CUC Genes during Embryogenesis

Homozygous single mutant lines of cuc3-101 and cuc3-105 were established to analyze the effect of the loss of CUC3 activity alone. Each mutant produced 12 and 2% of seedlings with cotyledons partially fused along one side, respectively (referred to as heart-shaped cotyledons; Figure 2A, Table 2). These

<table>
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<th>Class No.</th>
<th>Isolation No.</th>
<th>Gene and Allele No.</th>
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<sup>a</sup> Stop codon.  
<sup>b</sup> Isolated from an independent enhancer screening of cuc2-1.

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<th>Genotype (n)</th>
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<sup>a</sup> Frequencies of plants that lack primary shoot production among the cup-shaped seedlings at 10 DAG.  
<sup>b</sup> PCR-genotyped progeny of cuc1 cuc2/+.  
<sup>c</sup> PCR-genotyped progeny of cuc1 cuc2/+ cuc3/+ plants.

Table 1. Classification Mutant Alleles Identified by cuc2 Enhancer Screening

Table 2. Frequency of Seeding Phenotypes
cuc3-101 seedlings with heart-shaped cotyledons. (B) and (G) cuc1-1 cuc3-101 double mutant seedlings at 6 (B) and 10 d (C) after germination (DAG). Arrow in (C) indicates a shoot. (D) cuc1-1 cuc2-1 cuc3-101 triple mutant seedling derived from a cuc1-1 cuc2-1/cuc3-101/+ parent plant. (E) to (H) Shoot apices in cleared seedlings of Landsberg erecta (Ler) (E), cuc1-1 cuc3-101 (F), cuc2-1 cuc3-101 (G), and cuc1-1 cuc2-1 (H) at 2 DAG, showing the presence (E) to (G) or absence (H) of the shoot meristem. Bars = 50 µm.

frequencies were significantly higher than those in single mutants of strong cuc1 or cuc2 alleles.

To establish genetic relationships among the three CUC genes, we generated double and triple mutants of all possible combinations. The cuc2 cuc3 double mutant produced seedlings with cup-shaped cotyledons at a high frequency, and more than half of them failed to produce primary shoots (Table 2). By contrast, although the cuc1 cuc3 double mutant produced seedlings with a cup-shaped cotyledon at a frequency comparable to cuc2 cuc3, most of them formed a primary shoot (Figures 2B and 2C). Consistent with the occasional shoot formation in cuc1 cuc3 and cuc2 cuc3, we frequently observed an embryonic shoot meristem in the seedling apex of these genotypes (Figures 2E to 2G) in contrast with cuc1 cuc2, which never forms a shoot meristem (Figure 2H; Aida et al., 1997). In cuc1 cuc3 and cuc2 cuc3, however, production of the first pair of leaf primordia was significantly delayed compared with the wild type (Figures 2E to 2G). Because the subsequent growth of the mutant leaf primordia was similar to the wild type, this defect would most likely reflect a defect in embryonic shoot meristem initiation. All seedlings of cuc1/+ cuc2 cuc3, cuc1 cuc2/+ cuc3, and cuc1 cuc2 cuc3 exhibited the cup-shaped cotyledon phenotype and never formed a primary shoot (Figure 2D, Table 2; data not shown).

Taken together, these results suggest that CUC3 functions redundantly with CUC1 and CUC2 in shoot meristem formation and cotyledon separation during embryogenesis. The observed differences in severity of seedling phenotypes of each single and double mutant indicate distinct contributions of individual CUC genes in the processes (see Discussion).

CUC3 mRNA Expression in the Embryo

Expression pattern of CUC3 during embryogenesis has been reported by Vroemen et al. (2003), and we confirmed their results. In summary, the CUC3 mRNA is detected in the apical region of the early globular embryo (Figure 3A), becomes restricted to the L1 layer in a region between cotyledon primordia at the heart stage (Figures 3B to 3D), and later disappears from the shoot meristem and accumulates in cells along the boundary between cotyledon and the meristem (Figure 3E).

We next examined the effect of CUC1 and CUC2 activities on CUC3 expression. In cuc1-1 cuc2-1 double mutant embryos, CUC3 was detected in cells at the base of cup-shaped cotyledon primordia (Figure 3F, bottom panel) in contrast with the wild type showing the signal mainly in the L1 layer at the same stage (Figures 3D). We next asked whether ectopic CUC1 activity induces CUC3 expression using 35S:CUC1, in which several meristem-specific genes are ectopically expressed in cotyledons (Hibara et al., 2003). RT-PCR experiments detected no ectopic accumulation of CUC3 transcript in cotyledons of 35S:CUC1 seedlings (data not shown). These results suggest that the transcription of CUC3 does not primarily require CUC1 and CUC2 activities.

It has been shown that the expression of STM, which is detected in the presumptive embryonic shoot meristem in the wild type (Figure 3G), is completely missing in cuc1-1 cuc2-1 embryos (Aida et al., 1999). We examined the effect of cuc2 cuc3 and cuc1 cuc3 double mutations on STM expression. In cuc2-1 cuc3-101, we failed to detect STM expression at a high frequency (detected in 23 of 67 embryos; 34.3%), while a majority of cuc1-1 cuc3-101 embryos expressed STM (23 of 25 embryos;
92.0%). These frequencies were roughly correlated with those observed for primary shoot formation at the seedling stage (55.7% in cuc2-1 cuc3-101, n = 280; 94.7% in cuc1-1 cuc3-101, n = 206). When detected, expression of STM in cuc1 cuc3 or cuc2 cuc3 embryos tended to disappear specifically from the L1 layer of the presumptive shoot meristem (Figure 3H), suggesting that CUC3 promotes STM expression mainly in the L1 layer during embryogenesis. This is consistent with the observation that CUC3 expression in the wild type is transiently restricted to the L1 layer of the presumptive meristem.

The Roles of the CUC Genes in Axillary Meristem Formation

Previous analyses have shown that CUC1 and CUC2 are redundantly required for embryonic and adventitious shoot meristem formation (Aida et al., 1997; Daimon et al., 2003). Here, we characterized the roles of CUC1, CUC2, and CUC3 in postembryonic development. In the wild type, axillary meristems are formed at the axil of rosette and cauline leaves and develop branches (Figures 4A and 4C). On the other hand, the cuc3 single mutation disturbed branch formation at a low frequency, and this frequency was greatly enhanced in the cuc2 cuc3 double mutant. One of the most frequently observed phenotypes in these backgrounds is the lack of branch formation from the axil of rosette and cauline leaves (Figures 4B and 4D). Longitudinal sections revealed that such leaf axils showed no sign of axillary meristem formation (Figure 4E). In other cases, the cuc2 cuc3 double mutant showed a concaulescence phenotype, in which axillary shoots from cauline leaves were fused to the main stem (Figure 4F). The vascular bundle of fused axillary shoots splits from that of the main stem at the axil of the subtending cauline leaf, the position of which is similar to that of the wild type (Figures 4G and 4H). These observations suggest that the axillary shoot was initially formed properly at the leaf axil but grew for a while without separating from the main stem. In some cases, the axil of cauline leaves formed a single flower instead of an axillary shoot, and its pedicel fused with the cauline leaf. We refer to this phenotype as recaulescence (Figure 4I).

We scored the frequency of the axillary shoot defects produced from the secondary or tertiary shoots from cauline leaf axils in different cuc mutant combinations (Figures 5A and 5B). Axils of the cuc3 single mutant lacked tertiary shoots (i.e., axillary shoots from secondary shoots) at a low frequency (Figure 5A) and occasionally exhibited concaulescence or recaulescence phenotypes in the main and secondary shoots (Figure 5B). On the other hand, axils of cuc1 or cuc2 single mutants rarely show these phenotypes. Although cuc2 single mutation alone rarely affected axillary shoot development, it greatly enhanced the defects of cuc3 mutants: a majority of the cuc2 cuc3 double mutant axils either lacked axillary shoots or exhibited the concaulescence/recaulescence phenotypes. By contrast, the severity of axillary shoot phenotypes in the cuc1 cuc3 double mutant was the same as that of the cuc3 single mutant.

Double mutant combinations of cuc1 and cuc2 strong alleles cause complete lack of embryonic shoot meristem formation (Aida et al., 1997), preventing us from characterizing their phenotypes after the seedling stage. We therefore induced calli from cuc1-1 cuc2-1 hypocotyl and regenerated postembryonic
shoots (Aida et al., 1997). The regenerated shoots developed normal branches, indicating that the cuc1 cuc2 double mutation does not affect axillary shoot development (see Supplemental Figures 2A and 2B online). To obtain further support for the effect of cuc1 cuc2, we used two other genetic combinations. One is the double mutant of the weak cuc1-6 and the strong cuc2-1 alleles, which results in frequent production of postembryonic shoots from the seedling apex. The other is the triple mutant of cuc1-1 cuc2-1 as1-1. Because AS1 antagonizes a downstream pathway of CUC1 and CUC2, the as1 mutation partially suppresses the meristemless phenotype of cuc1 cuc2 seedlings (Hibara et al., 2003). Both genotypes produce relatively high frequencies of cup-shaped cotyledon phenotypes, indicating that the pathway involving CUC1 and CUC2 is effectively disrupted, though not completely. Neither cuc1-6 cuc2-1 nor cuc1-1 cuc2-1 as1-1 postembryonic shoot showed defects in axillary shoot formation (see Supplemental Figures 2C and 2D online).

Taken together, our data show that the cuc3 mutation disrupts normal axillary shoot development, and its effect is most prominent when combined with cuc2. The effect of cuc2 is only detectable in the presence of cuc3. On the other hand, we could not detect any effect of the cuc1 mutation on this process. These results indicate that CUC3 is a major regulator for axillary meristem initiation and separation of the meristem from the main stem. CUC2 is also involved in the processes, although its contribution is less prominent than that of CUC3.

Organ Separation in Postembryonic Development

We next examined the roles of CUC genes in postembryonic organ separation. Single mutants of cuc1-1, cuc2-1, and cuc3-101 showed no morphological defects in leaves. Among double mutant combinations, we frequently observed vegetative leaf fusions in cuc2-1 cuc3-101 (Figure 4J), while we never detected such a phenotype in cuc1-1 cuc3-101, cuc1-6 cuc2-1, and cuc1-1 cuc2-1 as1-1 (data not shown). Cauilne leaves of cuc2-1 cuc3-101 were occasionally fused to the inflorescence stem, causing strong distortion of the stem (Figure 4K). These results indicate that CUC2 and CUC3 are redundantly required for organ separation between leaves or between leaves and the stem. In the inflorescence stem of cuc3, pedicles are directed upward, forming a more acute angle with the stem compared with that of the wild type (see Supplemental Figures 3B and 3C and Supplemental Table 1 online). This phenotype was much more exaggerated in cuc2 cuc3, resulting in fusions of pedicels with the inflorescence stem (Figures 4L and 4M), indicating that CUC2 and CUC3 also promote separation of the pedicel from the stem. No such phenotype was observed in cuc1-1 cuc3-101, cuc1-6 cuc2-1, and cuc1-1 cuc2-1 as1-1 (data not shown; see Supplemental Figures 2C and 2D online). Fusions of pedicels to the stem or cauline leaves to the stem were not observed in the cuc1-1 cuc2-1 inflorescence induced from calli either (see Supplemental Figures 2A and 2B online).

In individual flowers, single mutants of cuc1 and cuc2 show subtle fusions of sepals and occasional stamen fusions, and the cuc1 cuc2 double mutant flowers on regenerated shoots show a strongly enhanced fusion phenotype of these floral organs (Aida et al., 1997). In the cuc3-101 single mutant, we detected fusion of stamens at a very low frequency and never detected sepal fusions (data not shown). Although cuc3-101 single mutation rarely caused fusions of individual floral organs, it moderately enhanced the fusion phenotypes of cuc1-1 and cuc2-1. These results indicate that CUC3 also contributes to floral organ separation but to a lesser degree than CUC1 or CUC2. The gynoecium of the cuc1-1 cuc2-1 double mutant shows reduced and malformed growth of septa and ovules, resulting in female sterility (Ishida et al., 2000). We did not detect any abnormality in the septon of cuc3, cuc1-1 cuc3-101, and cuc2-1 cuc3-101. In addition, fertility of these mutants was normal, indicating that they produce functionally normal ovules.

Expression of CUC3 in Postembryonic Development

We examined the expression pattern of CUC3 during postembryonic development. CUC3 mRNA accumulated at the boundaries between leaf primordia and the shoot meristem and between floral primordia and the inflorescence meristem (Figures 6A to 6D). These expression patterns are very similar.

Figure 5. Frequency of Axillary Shoot Phenotypes.

(A) The frequency of the lack of axillary shoots per cauline leaf axil in the main shoot (black bars) and in the secondary shoot (white bars). (B) The frequency of concaluescence and recalucescence phenotypes per cauline leaf axils in the main shoot (black bars, concaluescence phenotype; hatched bars, recalucescence phenotype) and in the secondary shoot (gray bars, concaluescence phenotype; white bars, recalucescence phenotype). At least 100 axils for each genotype were scored.
to those observed for CUC2 (Ishida et al., 2000). CUC1 is also expressed in a very similar manner in the inflorescence apex (Takada et al., 2001). In the vegetative meristem, on the other hand, the expression level of CUC1 is generally very low, and we could not conclusively define its expression domain in this tissue. A patch of CUC3 expression is detected within young flower primordia (arrows in Figures 6C and 6D).

The boundary between the meristem and leaf primordia will later form leaf axils. CUC3 was continuously expressed in the axils of rosette and cauline leaves until the initiation of axillary meristems (Figure 6E), consistent with its role in the formation of this tissue. After the initiation of axillary meristems, it is excluded from the center of the meristem (data not shown).

In the inflorescence, CUC3 mRNA was detected at the adaxial bases of pedicels or axillary buds (Figure 6F). Expression of CUC2 was also observed in the same regions (Figure 6G) but not CUC1 (data not shown). These results are in parallel with more predominant roles of CUC3 and CUC2 than CUC1 in separation of pedicels or axillary shoots from the stem. In developing flowers, CUC3 was detected in the boundaries of initiating sepal primordia, same as CUC1 and CUC2 (Figure 6H). At later stages, CUC3 expression became restricted to the epidermal cells of floral organ boundaries (Figure 6I) in contrast with CUC1 and CUC2 being expressed in the inner layer (Ishida et al., 2000; Takada et al., 2001). In the developing gynoecium, CUC3 expression was detected in L1 cells of the inner wall of valves and at the base of ovule primordia (Figure 6J). Unlike CUC1 and CUC2, expression of CUC3 was not detected in septum primordia (Figure 6J; Ishida et al., 2000; Takada et al., 2001). In developing ovules, we detected CUC3 expression at the base of the nucellus (Figure 6K).

Genetic Relation among the LAS and CUC Genes

The Arabidopsis LAS gene, which encodes a GRAS domain–containing putative transcriptional factor, is involved in the auxiliary meristem formation (Greb et al., 2003). The las mutant frequently fails to initiate vegetative axillary meristems from rosette leaf axils. In the inflorescence, it occasionally produces auxillary shoots fused to the stem from cauline leaf axils. LAS expression is detected at the boundaries between the meristem and leaf or between the meristem and flower primordia similar to CUC genes. We therefore investigated genetic interactions among LAS and CUC genes.
We obtained a T-DNA insertion allele of the LAS gene (*las-101*), which shows essentially the same axillary shoot phenotype as that reported for transposon insertion alleles (Greb et al., 2003; see Supplemental Figure 3A online). *las-101* occasionally lacks tertiary axillary shoots from the cauline leaf axil in the secondary stem (Figure 7A). In addition, we newly found that *las-101* exhibited upward growing pedicels, similar to the *cuc3* single mutant (see Supplemental Figures 3B to 3D and Supplemental Table 1 online). We then constructed double mutants between *las* and each of the *cuc* mutants. Inflorescence stems of *las cuc2* and

**Figure 7. Genetic Interactions among las and cuc Mutations.**

(A) The left panel shows the frequency of the lack of axillary shoots per cauline leaf axil in the main shoot (black bar) and in the secondary shoot (white bars). The right panel shows the frequency of concaulescence and recaulescence phenotypes per cauline leaf axils in the main shoot (black bars, concaulescence phenotype; recaulescence phenotype was not observed in the main shoot) and in the secondary shoot (gray bars, concaulescence phenotype; white bars, recaulescence phenotype). At least 100 axils for each genotype were counted.

(B) and (C) Concaulescence phenotype in *las-101 cuc2*-*3* (A) and *las-101 cuc3*-*105* (B). Arrows indicate fused parts.

(D) Fusion between pedicels and the stem in *las-101 cuc3*-*105*.

(E) Leaf fusion in *las-101 cuc3*-*105*. Arrows indicate fused parts.

(F) Seedling of the *las-101 cuc2*-*3 cuc3*-*105* triple mutant, showing extensive fusion of the leaf bases (arrow).

(G) Trumpet-shaped leaves in the *las-101 cuc3*-*105* double mutant (arrowheads).

(H) Scanning electron micrograph of trumpet-shaped leaf of *las-101 cuc3*-*105* viewed from the bottom. Bar = 200 µm.

(I) to (K) Close-up view of transverse sections of leaf petiole in the wild type (I) and the *las-101 cuc3*-*105* double mutant ([J] and [K]). The top side of each panel corresponds to the adaxial side. In the wild type, xylem cells (arrows) are located adaxially, while phloem cells (arrowheads) are positioned abaxially. In *las cuc3*, the petiole of trumpet-shaped leaves either completely lacks vascular bundles ([J] or contains only a single xylem tissue ([K], arrow). Bars = 40 µm.

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las cuc3 lost tertiary shoots at frequencies higher than that of las. Moreover, fusions of secondary or tertiary shoots to the stems were frequently observed in these double mutants (Figures 7A to 7C). By contrast, the las cuc1 double mutant does not result in enhanced lateral shoot phenotype compared with las (Figure 7A). In addition to the axillary shoot phenotype, las cuc3 but not las cuc2 or las cuc1 displayed fusion between pedicel and stem and between rosette leaves (Figures 7D and 7E; see Supplemental Table 2 online). All the phenotypes observed in las cuc3 were essentially the same as those of cuc2 cuc3, although their frequency and severity were milder than in cuc2 cuc3. Taken together, these results indicate overlapping roles of LAS and CUC3 in axillary meristem formation, separation of axillary shoots or flowers from the stem and separation of leaves. The triple mutant of las cuc2 cuc3 exhibited a leaf fusion phenotype much more severe than any of the double mutant combinations, such as cuc2 cuc3 (Figure 7F; see Supplemental Table 2 online), indicating that LAS is still active to promote leaf separation in the cuc2 cuc3 double mutant; conversely, CUC2 and CUC3 are active in the las mutant.

The las single mutant produced seedlings with heart-shaped cotyledons at a very low frequency (Table 2). Neither cuc1 nor cuc2 mutations enhanced this phenotype. However, the cuc3 mutation moderately increased the frequency of heart-shaped cotyledons in las (Table 2). In addition, las cuc3 produced cup-shaped cotyledons, which we did not find in the single mutants of las or cuc3. These results show that LAS is involved in cotyledon separation during embryogenesis. Consistent with this, LAS mRNA was detected in an intercotyledon region that will give rise to the shoot apical meristem and the cotyledon boundaries (see Supplemental Figures 4A to 4C online). LAS expression was also detected at the basal end of the embryonic root, including hypophysis derivatives (see Supplemental Figures 4B and 4C online). The cuc1 cuc2 double mutation specifically eliminates LAS expression in the intercotyledon region, while the expression in the root pole was not affected (see Supplemental Figure 4D online). These results indicate that CUC1 and CUC2 promote expression of LAS in the embryo shoot apex.

In addition to the above described phenotypes related to meristem and boundary development, we found a novel phenotype in leaves of las cuc3. In this mutant combination, we occasionally observed leaves with trumpet shape (Figures 7G and 7H; see Supplemental Table 2 online). In such leaves, trichomes are more prominently found in the outer surface (facing the petiole) than in the inner surface (away from the petiole). In histological sections of wild-type leaves, xylem tissues are situated adaxially, whereas phloem tissues are found on the abaxial side (Figure 7I). In las cuc3, trumpet-shaped leaves either completely lack vascular bundles or only contain the single xylem tissue but not phloem (Figures 7J and 7K). In less severely radIALIZED leaves, phloem cells were surrounded by xylem cells (data not shown). A very similar phenotype has been reported for mutations that cause leaf adaxialization, such as dominant alleles of the phabulousa or phavoluta mutants (McConnell and Barton, 1998; McConnell et al., 2001), indicating that the trumpet-shaped leaves of las cuc3 are adaxialized. These results indicate that CUC3 and LAS are also involved in the establishment of leaf polarity.

**DISCUSSION**

**Overlapping and Partially Distinct Functions of the CUC Genes Are Required for a Wide Range of Boundary and Meristem Formation in the Shoot**

In the plant life cycle, shoot meristem formation mainly occurs in two ways: the primary meristem formation during embryogenesis and axillary meristem formation in postembryonic development. Using cuc3 strong alleles that are newly isolated from our cuc2 enhancer screen, we confirmed the previous report that CUC3 acts redundantly with CUC1 and CUC2 during embryonic shoot meristem formation and cotyledon separation (Vroemen et al., 2003). More importantly, we found important roles of CUC2 and CUC3 in axillary meristem initiation during postembryonic development. The cuc3 single mutant occasionally lacks axillary meristems, and this phenotype is significantly enhanced by the cuc2 mutation. We have previously shown that CUC1 and CUC2 promote adventitious shoot formation from callus tissue (Daimon et al., 2003). Taken together, these results demonstrate that the three processes of shoot meristem initiation in Arabidopsis involves all or a subset of the three NAC proteins CUC1, CUC2, and CUC3.

The position of shoot meristem formation is tightly linked to the boundary of shoot lateral organs (Schmitz and Theres, 2005; Aida and Tasaka, 2006a, 2006b). The embryonic shoot meristem is initiated from the boundary between two cotyledon primordia. Likewise, axillary meristems are initiated from leaf axils, which develop from the boundary of leaf primordia and the shoot meristem. Our analysis shows that loss of CUC gene activities caused by various mutant combinations not only results in lack of shoot meristem formation but also causes severe organ fusions in cotyledon boundaries (where the embryonic shoot meristem develops), leaf/stem boundary (where the axillary meristem develops), and the boundary between the axillary shoot and the stem. Thus, the tight link between shoot meristem initiation and organ boundary formation seems to reflect the ability of the CUC genes for regulating both of the processes.

Comparisons of double mutants in all possible combinations reveal differences in individual contributions of the CUC genes. Double mutant combinations that carry wild-type alleles of CUC1 or CUC2 (i.e., cuc2 cuc3 and cuc1 cuc3, respectively) resulted in partial production of the embryonic meristem and separated cotyledons, while the cuc1 cuc2 double mutation completely disrupted both despite the presence of the wild-type CUC3 gene, indicating that the contribution of CUC1 and CUC2 during embryogenesis is greater than that of CUC3. Moreover, contribution of CUC2 is indicated to be greater than that of CUC1 because cuc2 cuc3 seedlings show stronger cotyledon and embryonic shoot meristem defects than cuc1 cuc3. Similar relationship is also found in floral organ separation, and CUC3 seems to play a minor role in this process compared with CUC1 and CUC2, as neither cuc1 cuc3 nor cuc2 cuc3 displays strong sepal fusion phenotype in contrast with cuc1 cuc2, which shows nearly complete fusion of sepals (Aida et al., 1997).

On the other hand, defects in axillary shoot formation and in separation of pedicels and the stem are frequently observed in cuc3 but not in the cuc1 or cuc2 single mutant. The phenotypes
in cuc3 are strongly enhanced by the cuc2 mutation but not by cuc1. These results indicate that CUC3 and CUC2 are major factors responsible for formation of the boundary between the stem and leaf and between the stem and pedicel and that the contribution of CUC3 is greater than that of CUC2. Although we did not detect any functional contribution of CUC1 in these processes, it has been reported that the cuc1 mutation enhances the branchless phenotype caused by the mutation in RAX1, a MYB transcription factor–encoding gene that promotes auxiliary meristem initiation (Keller et al., 2006). This result together with our data would indicate that CUC1 also promotes auxiliary meristem at least in the rax1 mutant background, although its contribution is much milder than that of CUC2 and CUC3.

What causes the observed differences in individual contributions of the CUC genes? One of the factors appears to be the difference in expression patterns. In the inflorescence apex, expression of CUC2 and CUC3 is initially detected in the boundary between the inflorescence meristem and young flower primordia and continues in the stem-pedicel boundary, consistent with their major role in stem-pedicel separation. By contrast, expression of CUC1, which does not affect the process, is detected initially at the boundary between the inflorescence meristem and flower primordia but not at later stages. It is thus suggested that the difference in the expression pattern is responsible for the different contribution of each CUC gene in stem-pedicel separation. A similar relationship is observed in axillary shoot development: expression of CUC2 and CUC3, which are main factors for axillary meristem formation and stem/auxiliary shoot separation, are readily detectable in leaf axis, whereas expression of CUC1 is hardly detectable. It is not clear, however, whether the CUC1 protein has a potential to promote this process when it is expressed artificially in the corresponding region using, for example, promoters of CUC2 or CUC3.

Differences in the protein property may also be responsible for the observed differences in individual CUC gene functions. In embryogenesis, expression of the three genes is observed in similar but not identical regions. The transcript of CUC3 is predominantly accumulated in the L1 layer of the embryo apex (Figures 3B and 3C; Vroemen et al., 2003), whereas those of CUC1 and CUC2 are predominant in the subprotodermal layers (Aida et al., 1999; Takada et al., 2001). The effect of CUC3 on STM expression is also predominant in the L1 layer, as revealed by specific loss of STM mRNA in the L1 layer in many of the cuc2 cuc3 or cuc1 cuc3 embryos (Figure 3H). These results raise the possibility that the mechanistic basis of the promotion of embryonic meristem formation and cotyledon separation by CUC3 is different from that of CUC1 and CUC2. In this regard, it is noteworthy that forced expression of CUC3 during embryogenesis under the 35S promoter does not cause any obvious seedling phenotype, while expression of CUC1 or CUC2 under the same promoter results in ectopic shoot meristem formation and lobing of the cotyledons (Takada et al., 2001; K. Hibara, K. Taoka, Y. Daimon, and M. Tasaka, unpublished results; T. Ishida and D.R. Smyth, personal communication). Moreover, the single mutation of cuc3 causes cotyledon fusion and axillary shoot phenotype at a frequency higher than that in the cuc1 or cuc2 single mutations, indicating that the loss of CUC3 is less well compensated by the other CUC genes than is the loss of either CUC1 or CUC2. These results suggest that the property of the CUC3 protein is different from that of CUC1 and CUC2. The proposed different protein function between CUC3 and CUC1/CUC2 is consistent with their phylogenetic relationship, in which CUC3 together with its potential monocot orthologs constitute a distinct subgroup from the one that includes CUC1 and CUC2 (Zimmermann and Wen, 2005).

Relationship of the LAS and CUC Genes

Previous data have shown the role of the LAS gene in axillary meristem formation, and the reported expression pattern of this gene also suggested that it may play wider roles in development of various shoot organ boundaries (Greb et al., 2003). Our genetic analysis demonstrated that LAS has indeed overlapping roles with CUC3 in various processes of postembryonic boundary formation, including axillary shoot formation, separation of pedicel and the stem, and leaf organ separation. Moreover, las enhanced the defect in cotyledon separation or shoot meristem initiation in several cuc mutant backgrounds, showing the involvement of LAS in boundary formation during embryogenesis. Consistently, LAS expression is detected in a region highly overlapping with that of the CUC genes and is downregulated in the cuc1 cuc2 double mutant. These results indicate that LAS acts downstream of CUC1 and CUC2. Taken together, our results show wider roles for LAS in the development of embryonic and postembryonic shoot organ boundaries.

We unexpectedly found that the las cuc3 double mutation occasionally results in the production of adaxialized leaves. This result indicates that CUC3 and LAS promote abaxial fate of leaf tissue and provides a first link between boundary specification and establishment of leaf polarity. Specification of leaf organ polarity appears to begin already in the anlagen as was revealed by promoter analysis of the abaxial-specific gene FILAMENTOUS FLOWER (Watanabe and Okada, 2003). Both CUC3 and LAS are expressed along the boundary between the shoot meristem and leaf primordium at least from the beginning of leaf primordium bulging, and this domain corresponds to the adaxial side of the primordium (Figure 6A; Greb et al., 2003). Classical surgical experiments have suggested that the shoot meristem produces a signal that promotes adaxial fate of the primordium, as an incision between the meristem and a leaf anlagen results in production of a radially symmetric adaxialized leaf (reviewed in Engstrom et al., 2004). One possibility is that the activities of CUC3 and LAS at the boundary region may weaken this putative signal.

METHODS

Plant Growth Conditions

Plants were grown on soil at 23°C under long-day condition (16 h of light). For examination of axillary shoot phenotypes, plants were grown under a short-day condition (10 h of light) for 30 to 35 d on soil and then were induced to flower under the long-day condition. For examination of seedling phenotypes, seeds were surface sterilized and germinated on Murashige and Skoog plates as described previously (Fukaki et al., 1996).
The cuc1-1 and cuc2-1 mutant have been described previously (Aida et al., 1997). The cuc1-5 mutant, isolated from the SLAT collection (Tissier et al., 1999) as the line SLAT 12_80_11, carries a dSpm insertion at 122 bp downstream of the ATG codon and was kindly provided by Robert Sablowski. The cuc2-3 mutant, originated from the Syngenta Arabidopsis Insertion Library collection (Sessions et al., 2002), carries a T-DNA insertion at 99 bp upstream of the ATG codon. The cuc2-2 mutation spontaneously arose from a cuc1-1 plant, resulting in duplication of the sequence GTTCAAAG within the second exon of CUC2 and a 745 bp insertion between them. Sequence analysis suggests that the insertion represents a novel nonautonomous transposon related to Tag1 (Tsay et al., 1993; M. Sato and M. Tasaka, unpublished results). The cuc3-105 mutants, carrying a T-DNA insertion in the second exon, was originated from the GABI-Kat collection (Rosso et al., 2003; accession number 302G09). The cuc1-13 and las-101 mutants are the T-DNA insertion lines SALK_006496 and SALK_000896 from the Salk Institute Genomic Analysis Laboratory collection (Alonso et al., 2003). cuc1-1, cuc2-1, and cuc2-2 are in Ler and cuc1-5, cuc1-13, cuc2-3, cuc3-105, and las-101 are in Col. Seedling phenotypes of cuc1-1 cuc2-2, cuc1-5 cuc3-3, and cuc1-13 cuc3-3 double mutants were essentially the same as that of cuc1-1 cuc2-2 (Aida et al., 1997), indicating that the newly isolated cuc1 and cuc2 insertion mutants represent strong alleles of cuc1 or cuc2. All mutants were subjected to detailed phenotypic analyses after at least three backcrosses to the wild type.

Mutant Isolation
Seeds of cuc2-1 were mutagenized in 0.4% EMS at room temperature for 8 h. M1 plants were grown and harvested individually. Independent 3500 M2 lines were screened for seedlings with the cup-shaped cotyledon phenotype or shoot meristemless phenotype on Murashige and Skoog plates. For phenotypic analyses, we used cuc3-101 after at least three backcrosses into Ler or cuc2-1.

Map-Based Cloning of CUC3
Plants from the line X84 were crossed to Col, and F2 seedlings with cup-shaped cotyledon phenotype were examined for recombinations between the mutation and PCR-based polymorphic markers. The genic sequences of At1g76420 (CUC3) of the lines X84, B24, E68, and fun3 were amplified by PCR using ExTag DNA polymerase (Takara). The resulting PCR products were directly sequenced using a dye terminator cycle sequencing kit and an ABI PRISM 3100 sequencer (Applied Biosystems).

Isolation of Ph CUC3
Phylogenetic analysis based on a partial amino acid sequence reported for Petunia x hybrida nam-like protein 16 (NH16; accession number AF510213) suggested that this protein represented a petunia ortholog of CUC3 and was thus designated Ph CUC3. To determine the full-length coding sequence of Ph CUC3, we designed gene-specific primers based on the partial sequence and performed 5’ rapid amplification of cDNA ends from cDNA derived from the inflorescence apex of P. hybrida using the GeneRac kit (Invitrogen). Phylogenetic analysis using the full-length protein sequences put Ph CUC3 into the same clade that includes CUC3, ZmCUC3, and Os CUC3 but not other reported NAC proteins (data not shown).

Complementation of the cuc3 Mutant
A 6.1-kb genomic fragment containing 3.8-kb 5′ and 0.5-kb 3′ sequences was subcloned into pBIN19AN, a binary vector modified from pBIN19. This construct was transformed into the Agrobacterium tumefaciens strain GV3101:pMP90 and then introduced into cuc2-3 cuc3-105 plants. T1 plants were selected for resistance to kanamycin. Seven out of seven T1 seedlings showed normal cotyledon morphology and shoot production.

Histological Analysis
In situ hybridization was performed as described by Takada et al. (2001). The CUC1, CUC2, and STM probes have been reported by Takada et al. (2001), Aida et al. (1999), and Long et al. (1996), respectively. The CUC3 probe was prepared from a CUC3 1005-bp cDNA fragment containing the full-length open reading frame. Hybridization was performed at 45 to 50°C. Western Blue (Promega) was used as a substrate for signal detection. Scanning electron microscopy was performed as described by Aida et al. (1999). For visualization of vasculature, tissues were fixed in ethanol:acetic acid (9:1) solution at room temperature. After rehydration in 90% ethanol for 30 min and then 70% ethanol overnight, seedlings were observed. For histological sections, seedlings were fixed in formalin-acetic acid-alcohol overnight at 4°C, embedded in Technovit 7100 (Heraeus Kulzer), and cut with a microtome. Sections were stained with toluidine blue.

Accession Numbers
Sequence data of genes described in this article can be found in the Arabidopsis Genome Initiative, DDBJ/EMBL/GenBank, or Rice Annotation Project data libraries under the following accession numbers: CUC1 (At1g51570), CUC2 (At5g53950), CUC3 (At1g76420), LAS (At1g55580), STM (At1g62360), BrRm (At2g46020), GNOM (At1g13980), Ph CUC3 (AB255374), and Os CUC3 (Os08g0511200).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Sequence Alignments of CUC-Type NAC Proteins and the Genomic Structure of the CUC Genes.
Supplemental Figure 2. Mutations in CUC1 Have Little Effect on Axillary Shoot Formation.
Supplemental Figure 3. Inflorescence Phenotype of cuc3 and las.
Supplemental Figure 4. Expression Patterns of LAS mRNA in the Embryo.
Supplemental Table 1. Pedicel Angle in cuc and las Mutants.
Supplemental Table 2. Occurrence of Leaf Fusion and Trumpet-Shaped Leaf in Different Genetic Backgrounds.

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