The **CUP-SHAPED COTYLEDON1** gene of *Arabidopsis* regulates shoot apical meristem formation

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**SUMMARY**

In higher plants, molecular mechanisms regulating shoot apical meristem (SAM) formation and organ separation are largely unknown. The CUC1 (**CUP-SHAPED COTYLEDON1**) and CUC2 are functionally redundant genes that are involved in these processes. We cloned the CUC1 gene by a map-based approach, and found that it encodes a NAC-domain protein highly homologous to CUC2. CUC1 mRNA was detected in the presumptive SAM during embryogenesis, and at the boundaries between floral organ primordia. Surprisingly, overexpression of CUC1 was sufficient to induce adventitious shoots on the adaxial surface of cotyledons. Expression analyses in the overexressor and in loss-of-function mutants suggest that CUC1 acts upstream of the SHOOT MERISTEMLESS gene.

Key words: CUC, Shoot apical meristem, Organ separation, NAC, *Arabidopsis thaliana*

**INTRODUCTION**

The shoot apical meristem (SAM) is essential for the development of higher plants, because it generates most aerial parts after germination. In dicotyledonous plants, the embryonic SAM is formed between two cotyledon primordia during embryogenesis, and in postembryonic development, the SAM continuously produces stems and lateral organs (leaves and floral organs) in a regular pattern. In *Arabidopsis*, several mutations that affect SAM formation have been identified; shoot meristemless (stm), cup-shaped cotyledon1 (cuc1) cuc2 double mutants and wuschel (wus) are defective in embryonic SAM formation (Barton and Poethig, 1993; Endrizzi et al., 1996; Laux et al., 1996; Long et al., 1996; Aida et al., 1997; Mayer et al., 1998; Aida et al., 1999). The **STM** gene encodes a homeodomain protein of the KNOTTED1 class and its mRNA accumulates in cells predicted to form the embryonic SAM (Long et al., 1996). **KNOTTED1**-like homeobox genes are thought to be involved in SAM formation and its maintenance; the **KNOTTED1** gene of maize and a related *Arabidopsis* gene, KNAT1, are expressed in undifferentiated cells in the meristem and downregulated from leaf primordia (Jackson et al., 1994; Lincoln et al., 1994). Transgenic plants expressing **KNOTTED1** or KNAT1 under the control of the cauliflower mosaic virus 35S promoter show abnormal leaves and adventitious SAMs on the adaxial surface of the leaves (Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996). To date, only **KNOTTED1**-type homeobox genes and a cytokinin-synthesizing gene have been reported to induce adventitious shoots when they are expressed ectopically (Estruch et al., 1991; Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996).

**WUS** encodes a homeodomain protein of a novel class distinct from the KNOTTED1 class and is expressed in a group of cells underneath the stem cells of the SAM. **WUS** is postulated to affect stem cell fate in a non-cell-autonomous manner (Mayer et al., 1998). **WUS** and **STM** seem to act at different regulatory levels in SAM development, because the initiation of **WUS** expression is independent of **STM** activity, and because **WUS** is expressed in a small subdomain of the SAM (Mayer et al., 1998).

**cuc1 cuc2** double mutant seedlings completely lack an embryonic SAM, and two cotyledons are fused along both edges to form one cup-shaped structure (Aida et al., 1997). In addition, adventitious shoots regenerated from mutant calli form flowers in which sepals and stamens are severely fused (Aida et al., 1997). However, **cuc1** or **cuc2** single mutant seedlings look normal except for occasional seedlings with fused cotyledons in one of their sides (Aida et al., 1997). In each single mutant flower, only weak fusion of sepals and stamens is observed (Aida et al., 1997). These observations indicate that **CUC1** and **CUC2** are functionally redundant genes that are required for embryonic SAM development and for keeping cotyledons and floral organs from fusing with each other. **CUC1** and **CUC2** are thought to promote embryonic SAM formation through transcriptional activation of **STM**, because the accumulation of **STM** mRNA is blocked in **cuc1 cuc2** double mutant embryos (Aida et al., 1999). **CUC2** is
expressed in the presumptive SAM between two cotyledon primordia (Aida et al., 1999). It is also expressed at the boundaries between the vegetative meristem and leaf primordia, between the inflorescence and floral meristems and between floral organs (Ishida et al., 2000).

**CUC2** is highly homologous to the petunia nam gene that is required for floral organ development, cotyledon separation and embryonic SAM formation (Sourer et al., 1996; Aida et al., 1997). N-terminal halves of CUC2 and NAM contain highly conserved sequences called the NAC domain. CUC2 and NAM also share several homologous sequences in their C-terminal halves (Aida et al., 1997). NAC-domain encoding genes (NAC genes) constitute a large gene family and are not found in organisms other than plants, suggesting that they may have unique roles in plant development. Recently, several NAC genes have been isolated and characterized in many plant species (Sourer et al., 1996; John et al., 1997; Sablowski and Meyerowitz, 1998; Ruiz-Medrano et al., 1999; Xie et al., 1999). In Arabidopsis, NAP was isolated as an immediate target of the floral organ identity gene STM (Ishida et al., 2000). It is also expressed at the SAM formation and function is largely unknown.

In this study, we isolated the CUC1 gene by a map-based approach. CUC1 encodes a NAC-domain protein highly homologous to CUC2 and NAM. In situ hybridization experiments showed that CUC1 is expressed in cells predicted to form the embryonic SAM and at the boundaries between floral organs. CUC1 transcripts were also detected in cuc1, cuc2 and stm mutant backgrounds. Surprisingly, transformants ectopically expressing CUC1 under the control of the cauliflower mosaic virus 35S promoter had lobed cotyledons and adventitious shoots on the adaxial side of the cotyledons. STM mRNA was ectopically expressed in these cotyledons. These results suggest that CUC1 functions upstream of STM and regulates SAM formation during Arabidopsis embryogenesis.

**MATERIALS AND METHODS**

**Mutant isolation and alleleism tests**

cuc2 seeds (ecotype Landsberg erecta [Ler]; Aida et al., 1997) were mutagenized by incubating in 0.4% EMS for 8 hours. Individual M1 plants were grown and harvested independently. Independent 2200 M2 lines were screened for seedlings with the cup-shaped cotyledon phenotype. To determine alleleism, cuc1-1/cuc2/cuc2 plants were crossed to new mutant lines that segregate cup-shaped cotyledon seedlings.

**Mapping of the CUC1 gene**

cuc1-1/cuc1-1/cuc2/cuc2 plants (ecotype Ler) were crossed to wild-type plants (ecotype Col), and cuc1-1/cuc1-1/cuc2/cuc2 plants (F1) were obtained. Among the progeny of the F1 plants, 972 of cuc1-1/cuc1-1/cuc2/cuc2 double mutant seedlings were reexamined for recombinations between the CUC1 locus and PCR markers in chromosome 3. Degenerate primers used to amplify NAC genes from the YAC clone were PPGFHPI (5’-cccccgggttt[a/g]cggggg[a/g]a[g]a[g]a[g]ggttg-[c/t]-3’ [i: inosine]) and WVMMHEYLR (5’-a[a/g]cggggg[a/g]a[g]a[g]a[g]a[g]ggtttg-3’)-1 (5’-tagtgccgtcaaggctag-3’). Amplified fragments were sequenced directly. By searching databases, both AtNAC2 and AtNAC3 were mapped to the same BAC clone MJK13. Exon-intron structures of AtNAC2 and AtNAC3 were determined by reverse transcription PCR (RT-PCR). Physical map data of chromosome 3 were obtained from a sequence database developed by Kazusa DNA Research Institute. YAC clones were provided by the Arabidopsis Biological Resource Center (ABRC).

**Cloning of the CUC1 gene**

A genomic library of Arabidopsis (Columbia [Col] ecotype) in EMBL3 SP6/T7 (Clontech) was screened with a probe corresponding to the NAC-domain encoding region (NAC box) of CUC2, as described by Aida et al. (1997). A 3826-bp HindIII-Xhol fragment, which was strongly hybridized with the CUC2 probe, was subcloned into pBluescript II SK vector (Stratagene) and sequenced. CUC1 cDNA was obtained by using RT-PCR with primers X28-2 (5’-aaaaatctttcttggtgccacaagt-g-3’) and P4-5 (5’-gtgccagacgccgctggttcagaggag-3’). Both 5’ and 3’ ends of CUC1 cDNA were determined by using 5’ and 3’ rapid amplification of cDNA ends (RACE) system (Gibco-BRL). The longest cDNA identified had 1163 nucleotides.

**Complementation of the cuc mutant**

The 3.8-kb fragment containing the CUC1 gene was subcloned into pBIN19 and used to transform cuc1-1/cuc1-1/cuc2/+ plants. Genotypes of the CUC1 and CUC2 loci in the transgenic plants were determined by PCR. To detect the cuc1-1 mutation, we developed a dCAPS marker (Neff et al., 1998). Primers used for the dCAPS analysis were mCUC1-1 (5’-aactactctccgctgattcg-3’) and ANAC1-5 (5’-attggctcaagggctaca-3’). Endogenous CUC1 DNAs were obtained by PCR with primers ANAC1-2 (5’-taggctaggtagggacaagt-3’) and P4-17 (5’-aaactactctcaagaactgtc-3’; located outside the 3.8-kb region). These PCR products were reamplified with the dCAPS primers, and amplified fragments were digested with HincII, which produces a 105-bp fragment for the Ler allele and 86- and 19-bp fragments for the cuc1-1 allele. The cuc2 mutation was detected with primers CUP1B-1 (5’-cgagagtaaaaagtagcttca-3’) and Tag1-4 (5’-ctcagatggtgctggtt-3’), while the Ler allele of CUC2 was detected with primers CUP1B-1 and CUP1B-4 (5’-tccatacatataagcctc-3’).

**Sequence analyses and database searches**

Phylogenetic trees and alignments were constructed by using ClustalX version 10 (Software Development Co, Ltd). Database searches were performed at the National Center for Biotechnology Information by using the BLAST network service. The sequence data of the CUC1, AtNAC2, and AtNAC3 cDNAs have been deposited in the GenBank database with accession numbers AB049069, AB049070, and AB049071, respectively.

**RT-PCR analysis**

RNA was prepared using the Qiagen RNEasy kit (Qiagen). First strand cDNA was synthesized by using SuperScript Preamplification system (Gibco-BRL). One microgram of total RNA was treated with 1 Unit of amplification grade DNase I (Gibco-BRL) and first strand cDNA was synthesized by using SuperScript Preamplification system (Gibco-BRL). The longest cDNA identified had 1163 nucleotides. Phylogenetic trees and alignments were constructed by using ClustalX version 10 (Software Development Co, Ltd). Database searches were performed at the National Center for Biotechnology Information by using the BLAST network service. The sequence data of the CUC1, AtNAC2, and AtNAC3 cDNAs have been deposited in the GenBank database with accession numbers AB049069, AB049070, and AB049071, respectively.
Because treated total RNAs were reverse transcribed by using the RNA map kit (GeneHunter) in a 10 μl reaction as described by Aida et al. (Aida et al., 1997), and one tenth of the reaction were used as a template for PCR.

In situ hybridization

In situ hybridization was performed as described previously (Ishida et al., 2000) with the following modifications. Embryos were fixed in 4% paraformaldehyde in phosphate buffer, pH 7.0, overnight at 4°C. Seedlings and flowering buds were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) for 4 hours at 25°C. The template for CUC1 sense or antisense probes was a 453-bp region of CUC1 cDNA excluding the NAC box. This 453-bp region was amplified by PCR using P4-3 (5'-taaagcttgtctgaaaagcggcgtag-3') and P4-5 oligonucleotides, and subcloned into pBluescript II SK vector (Stratagene). Hybridization was performed at 45°C. Western Blue (Promega) was used as the substrate for the detection.

Construction of transgenic plants

35S::CUC1

The coding region of CUC1 cDNA was amplified by RT-PCR using primers X28-2 and P4-5 and subcloned between the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator in the binary vector pBI121 (Clontech).

CUC1:GUS fusion construct

0.59-kb region downstream of the translational stop was removed from the 3.8-kb CUC1 fragment, and the stop codon was replaced by a glycine-linker sequence. This CUC1 fragment was subcloned upstream of the GUS coding region in pBI101 (Clontech). The resulting construct contained a 1.7-kb region upstream of the CUC1 translational start site and a 1.5-kb coding region fused in-frame to the uidA gene.

Plant transformation

Arabidopsis thaliana (Ler ecotype) plants were transformed using the vacuum infiltration method (Bekhtold et al., 1993) with Agrobacterium strain MP90. Transgenic plants (T1) were screened on agar plates containing kanamycin (50 μg/ml). For RT-PCR analyses, RNAs were extracted from next progenies (T2) of each T1 transformant.

Histological analysis

For histological sections, tissues were fixed in 4% paraformaldehyde in phosphate buffer, pH 6.8, overnight at 4°C, embedded in Technovit 7100 (Heraeus Kulzer), cut with a microtome and stained with toluidine blue. To detect GUS activity, tissues were stained in a staining solution (1.9 mM X-Gluc, 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6 and 0.3% Triton X-100) at 37°C for 12 hours. Stained plants were cleared as described by Aida et al. (Aida et al., 1997). SEM analyses were done as described by Smyth et al. (Smyth et al., 1990).

RESULTS

Molecular cloning of CUC1

Because CUC1 and CUC2 are functionally redundant, CUC1 was expected to contain a NAC domain similar to that of CUC2 (Aida et al., 1997). To isolate new CUC2-like NAC genes, an Arabidopsis genomic library was screened with the probe of the NAC box of CUC2 at high stringency. Among 16 positive clones, four clones did not contain the genomic region of CUC2. In Southern hybridization experiments, one of the four clones was strongly hybridized with the NAC box of CUC2. It contained a predicted open reading frame that encoded a novel NAC-domain protein of 310 amino acids (Fig. 1B). We named this gene AtNAC1.

In parallel with the above experiment, we tried to clone the CUC1 gene using a map-based approach. First, the CUC1 locus was mapped to a single YAC clone, CIC5H8, in chromosome 3 (Fig. 1A). Next, degenerate primers were designed to detect NAC genes. Three genome fragments were amplified from the YAC clone with these primers (data not shown). These PCR products were sequenced, and one of the three bands was found to correspond to AtNAC1. The other two bands also contained parts of other NAC-box sequences. These clones were named AtNAC2 and AtNAC3. We then determined the map position of the CUC1 locus in detail by mapping recombination break points and found that both CUC1 and AtNAC1 mapped to a 51.8-kb region (Fig. 1A). To examine whether AtNAC1 corresponds to CUC1, we sequenced the AtNAC1 locus in the cuc1 mutant background. Although only one cuc1 allele (cuc1-1) was used in previous studies, we have isolated three new cuc1 alleles (cuc1-2, cuc1-3 and cuc1-4) in a screen for enhancer mutants of cuc2 (see Materials and Methods). We found that all four cuc1 alleles had mutated sequences in the AtNAC1 locus (Fig. 1B; see below). To confirm that AtNAC1 is identical to CUC1, a 3.8 kb-genomic fragment containing the AtNAC1 gene was transformed into cuc1-1/cuc1-1 cuc2/+ plants. Eleven kanamycin-resistant transformants were generated, and all of them developed the embryonic SAM (Fig. 1C). We found that two of the 11 transformants had cuc1-1 cuc2 double mutations, indicating that AtNAC1 complemented the cuc phenotype. We therefore conclude that AtNAC1 is the CUC1 gene.

Structure of the CUC1 gene

The NAC domain of CUC1 was similar to that of CUC2 and NAM, while NAC domains of AtNAC2 and AtNAC3 were similar to those of ATAF1, ATAF2, GRAB1 and NAP (Fig. 1D,E). Moreover, CUC1, CUC2 and NAM had conserved sequences in the C-terminal half (Fig. 1B,F). These conserved sequences were not found in AtNAC2, AtNAC3, ATAF1, ATAF2 and NAP (data not shown). cuc1-1 and cuc1-4 contained missense mutations that altered conserved amino acids within the NAC domain (Fig. 1B). In cuc1-3, a single nucleotide substitution created a stop codon near the C-terminal end of the CUC1 protein. Interestingly, the putative mutant protein in cuc1-3 only lacks five amino acids in the C-terminal end. This C-terminal sequence was conserved among several NAC-domain proteins (Fig. 1G). Although the biochemical functions of CUC proteins are unknown, these results suggest that these amino acids are important for the function of the CUC1 protein. In cuc1-2, the 3'-intron-exon boundary of the first intron was changed from AG:AG to AA:AG (Fig. 1B). RT-PCR analyses revealed that cuc1-2 accumulated long messenger RNA that failed to remove the first intron (data not shown). This splicing defect results in a translational stop after four codons within the intron.

Expression patterns of CUC1

CUC1 mRNA was detected in inflorescence stems, rosette leaves, aerial parts of seedlings, flowers, floral buds and roots by using quantitative RT-PCR method (Fig. 2A). This indicates that CUC1 is expressed more widely than CUC2.

CUC1 and SAM formation in Arabidopsis
To examine the spatial and temporal expression of the \textit{CUC1} gene in wild-type development, we performed in situ hybridization experiments. In wild-type embryos, \textit{CUC1} mRNA was first detected in globular-stage embryos, where it was found in two separate regions within the apical part (Fig. 3A, arrows). In early-heart-stage embryos, \textit{CUC1} mRNA was detected between the two cotyledon primordia (Fig. 3B, arrowhead); \textit{CUC1} was expressed in a stripe across the top half of embryos (Fig. 3C). From histological analysis, cells at the center of this region are predicted to give rise to the embryonic SAM (Barton and Poethig, 1993). The transcripts were only weakly detected in the L1 layer (Fig. 3B,C). In peripheral regions of early-heart-stage embryos, \textit{CUC1} expression extended below the O circle (Fig. 3C; West and Harada, 1993).

These expression patterns of \textit{CUC1} overlapped with those of \textit{CUC2} and \textit{STM} (Long et al., 1996; Aida et al., 1999).
heart-stage embryos, CUC1 transcripts started to disappear from the central SAM regions (Fig. 3D). In bending-cotyledon-stage embryos, CUC1 was detected at the boundaries between cotyledon primordia and the SAM (Fig. 3E, arrows). In mature-stage embryos, CUC1 transcripts were detected around slight bulges where the first pair of leaf primordia would be formed (Fig. 3F,G). After germination, CUC1 transcripts were only weakly detected in the vegetative meristem (data not shown).

In inflorescence shoots, CUC1 seemed to be expressed at the boundary regions between the inflorescence meristem (IM) and stage 1 young floral meristems (FMs; Fig. 4A, arrowhead). CUC1 mRNA was also detected in the axillary meristems (Fig. 4B, arrow). In stage 4-5 FMs, CUC1 transcripts were detected at the boundaries between the third and fourth whorls as well as at the boundaries between sepal primordia and the central dome of the FMs (Fig. 4B). In stage 7 FMs, the transcripts were detected between sepal and petal primordia, between petal and stamen primordia and between stamen and gynoecium primordia (Fig. 4D). CUC1 was detected at the boundaries of individual sepal primordia and of individual stamen primordia in transverse sections of FMs (Fig. 4F,G). In the second whorl, CUC1 was detected in cells surrounding the petal primordia (data not shown). In stage 7 FMs, transcripts of CUC1 appeared in septum primordia and in an adaxial part of stamen primordia (Fig. 4D,H). In stage 10-11 FMs, CUC1 mRNA was found in part of the ovules (Fig. 4E). CUC1 was also detected at the boundaries between the two locules of each theca in anthers (data not shown). Control experiments using a sense probe of CUC1 resulted in no signal above background (data not shown).

CUC1 expression was also detected using transgenic Arabidopsis carrying a CUC1 genomic fragment fused in-
frame to the *uidA* gene which encodes the β-glucuronidase (GUS) protein (see Materials and Methods). In inflorescences, CUC1::GUS expression patterns were similar to those observed in in situ hybridization experiments (Fig. 4C). In embryos, CUC1::GUS expression was very weak and could not be analyzed precisely (data not shown).

In summary, CUC1 transcripts were detected in cells that give rise to the SAM during embryogenesis, and in reproductive tissues where they were detected between the IM and FMs as well as at the boundaries of floral organ primordia. These expression patterns of CUC1 overlapped with those of CUC2 (Aida et al., 1999; Ishida et al., 2000).

**CUC1 expression in mutant backgrounds**

To investigate whether CUC1 expression is regulated by CUC1, CUC2 and STM, we examined the expression of CUC1 in *cuc1-1*, *cuc2* and *stm-1* mutant backgrounds. CUC1 mRNA was detected at normal levels in *cuc1-1* and *cuc2* single mutants by using quantitative RT-PCR (Fig. 2B). In addition, by in situ hybridization, CUC1 transcripts were detected in *cuc1-1* and *cuc2* inflorescences in a normal expression pattern as seen in wild-type plants (Fig. 4L). In *stm-1/+* siliques, 50 of 50 embryos expressed CUC1, indicating that CUC1 was expressed in *stm-1* mutants. In mature *stm-1* embryos, CUC1 was expressed throughout the boundary region between cotyledon primordia (Fig. 3H). These results suggest that CUC1 expression does not need the activities of CUC1, CUC2 and STM. In *stm-1* embryos, CUC1 was not downregulated in the center of the boundary region (data not shown). However, spatial expression of CUC1 in *stm-1* was less disturbed than that of CUC2 (Aida et al., 1999).

**Overexpression of CUC1**

To further examine the role of CUC1 in *Arabidopsis* development, the CUC1 coding region was fused to the 35S promoter in a sense direction, and the chimeric gene was transformed into *Arabidopsis*. Two of 16 primary transformants (T1) showed fused cotyledons and fused floral organs (sepal and stamens) in the next T2 generation (data not shown). In these lines, expression levels of CUC1 were reduced by co-suppression (data not shown).

The remaining 14 kanamycin-resistant transformants showed a novel phenotype in the T2 generation (Fig. 5). Among them, six independent lines were analyzed in detail. In all six lines, expression levels of CUC1 were increased (Fig. 6A). In these 35S::CUC1 plants, lateral margins of cotyledons were split to form a lobed structure (Fig. 5A,B). This phenotype was observed in all 6 lines, although the severity varied among these lines (Table 1). In severe lines, cotyledons were much smaller than those of wild type (Fig. 5E).

The morphology of rosette leaves was also altered in 35S::CUC1. These leaves seemed to consist of two leaves fused to each other along one lateral margin of the petioles and leaf blades (Fig. 5C), as two primary vascular strands were observed in these leaves (data not shown).

The most interesting phenotype of 35S::CUC1 was the formation of adventitious shoots on the adaxial surface of the lobed cotyledons (Fig. 5B,E). These shoots initially formed rosette leaves, and some of them subsequently developed to form inflorescences and seeds (data not shown). This ectopic

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**Table 1. Phenotypes of 35S::CUC1 lines**

<table>
<thead>
<tr>
<th>Line number</th>
<th>Lobed cotyledons (%)</th>
<th>Cotyledons with adventitious shoots (%)</th>
<th>Fused leaves (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1-6 (n=105)</td>
<td>54.3</td>
<td>5.7</td>
<td>3.8</td>
</tr>
<tr>
<td>S2-6 (n=84)</td>
<td>91.7</td>
<td>29.8</td>
<td>6.0</td>
</tr>
<tr>
<td>S3-7 (n=80)</td>
<td>72.5</td>
<td>43.8</td>
<td>7.5</td>
</tr>
<tr>
<td>S4-11 (n=77)</td>
<td>11.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>S5-10 (n=46)</td>
<td>50.0</td>
<td>19.7</td>
<td>6.5</td>
</tr>
<tr>
<td>S6-10 (n=90)</td>
<td>42.2</td>
<td>6.7</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Two-week-old plants were scored for the above phenotypes.
Adventitious shoot formation was clearly visible, whereas was ectopically expressed in cotyledons of 35S::CUC1. This was not expressed in these cotyledons. These results suggest plants by quantitative RT-PCR (Fig. 6B).

To test this hypothesis, we examined the expression of embryonic SAM formation (Fig. 3H; Aida et al., 1999). To test this hypothesis, we examined the expression of embryonic SAM formation (Fig. 3H; Aida et al., 1999). To test this hypothesis, we examined the expression of embryonic SAM formation (Fig. 3H; Aida et al., 1999). To test this hypothesis, we examined the expression of embryonic SAM formation (Fig. 3H; Aida et al., 1999). To test this hypothesis, we examined the expression of embryonic SAM formation (Fig. 3H; Aida et al., 1999).

Expression analyses in 35S::CUC1. (A) Quantitative RT-PCR analysis of CUC1 expression in 35S::CUC1 lines. RNAs were isolated from rosette leaves of 35S::CUC1 (S1-6, S2-6, S3-7, S4-11, S5-10 and S6-10) and Ler (Ler leaf), and from floral buds of Ler (Ler bud). (B) Quantitative RT-PCR analysis of CUC2 and STM expression in 35S::CUC1 cotyledons (35S::CUC1), Ler cotyledons (Ler) and Ler floral buds (bud). ACT8 was used as an internal control (An et al., 1996).

Shoot formation correlated with the severity of the lobed cotyledon phenotype (Table 1). Near the sinus-like regions of the lobed cotyledons the epidermis cells were smaller and rounder than in wild type (Fig. 5E,G). In severe lines, these epidermal cells were distributed in a broad zone connecting two sinus-like regions of the lobed cotyledon (Fig. 5E). In this zone, differentiated cells such as stomata and pavement cells were not found (Fig. 5G). Many meristem-like structures and leaf primordia were observed on the adaxial side in this zone, and several adventitious shoots often developed simultaneously from adjacent positions (Fig. 5G). In 35S::CUC1, adventitious shoots were always formed near vascular strands of lobed cotyledons (Fig. 5H).

Ectopic shoots were also formed on the adaxial surface of true leaves, although the frequency was much lower compared to that of ectopic shoot formation on the cotyledons (data not shown). Expression analyses in cuc1-1 cuc2 and stm-1 mutants suggested that CUC1 acts upstream of STM and regulates embryonic SAM formation (Fig. 3H; Aida et al., 1999). To test this hypothesis, we examined the expression of STM and CUC2 in 35S::CUC1 plants by quantitative RT-PCR (Fig. 6B). STM was ectopically expressed in cotyledons of 35S::CUC1 before the adventitious shoots were clearly visible, whereas CUC2 was not expressed in these cotyledons. These results suggest that CUC1 is able to induce STM expression in the absence of CUC2.

DISCUSSION

CUC1 encodes a NAC domain protein

In this study, we have shown that CUC1 encodes a NAC-domain protein highly homologous to CUC2. The phylogenetic analyses suggest that the NAC gene family can be divided into at least two subfamilies: CUC1/CUC2-like NAC genes (CUC1, CUC2 and nam) and ATAF-like NAC genes (ATAF, NAP, AtNAC2 and AtNAC3). The CUC1/CUC2-like NAC genes also share homologous sequences in the 3’ region (this study; Souer et al., 1996; Aida et al., 1997). CUC1, CUC2 and nam are expressed at the boundaries between cotyledonal primordia and between floral organs (this study; Souer et al., 1996; Aida et al., 1999; Ishida et al., 2000). We speculate that CUC1/CUC2-like NAC genes are specifically involved in SAM formation and separation of cotyledons and floral organs. We have identified three other CUC1/CUC2-like NAC genes (AtNAC4, AtNAC5 and AtNAC6) from Arabidopsis genome databases (Fig. 1D). We are now screening for T-DNA insertion lines disrupting these loci.

CUC1 is expressed in the presumptive SAM and at the boundaries of all floral organs

CUC1 mRNA was detected in cells that are predicted to form the embryonic SAM, suggesting that CUC1 functions within these cells and regulates SAM formation during Arabidopsis embryogenesis. In contrast, CUC1 was not detected in the center of the vegetative or the inflorescence meristem, but was detected at the boundary region between the inflorescence meristem and young floral primordia. CUC1 expression in these postembryonic SAMs may not have any function in Arabidopsis development, because CUC genes are not essential for leaf and floral meristem formation (Aida et al., 1997). Alternatively, CUC genes may have redundant functions in these postembryonic SAMs with other NAC genes.

In cuc1-1 mutants, sepals are often fused, and sometimes stamens and cotyledons are also fused. Consistently, CUC1 was expressed at the boundaries between primordia of cotyledons, sepals and stamens. These results suggest that the function of CUC1 is to inhibit the growth of cells at the boundaries between primordia of cotyledons, sepals and stamens to keep these organs from fusing with each other. However, fused petal phenotypes are not observed in cuc1-1 and cuc2 mutants, and CUC1 and CUC2 are expressed in cells surrounding the petal primordia (Aida et al., 1997; Ishida et al., 2000). In Arabidopsis, petal primordia are formed at relatively distant positions from each other (Smyth et al., 1990). Thus, it is likely that CUC1 and CUC2 separate such organ primordia (cotyledons, sepals and stamens) that develop simultaneously from adjacent positions. It is possible that other NAC genes or other regulatory mechanisms are involved in the separation of petal primordia.

In cuc1-1 mutants, partially unfused septa are formed in siliques (Ishida et al., 2000). This phenotype is consistent with the expression of CUC1 in septum primordia. CUC2 is also expressed in septum primordia, and unfused septa are observed in cuc2 mutants (Ishida et al., 2000).

Redundancy between CUC1 and CUC2

CUC1 and CUC2 are highly homologous to each other, and regulate the same developmental processes. CUC1 was expressed in cuc2 mutants, and CUC2 was expressed in cuc1-1 mutants. Overexpression of CUC1 induced ectopic STM expression in the absence of CUC2 expression. These results suggest that the function of either CUC1 or CUC2 is sufficient for embryonic SAM formation and that CUC1 and CUC2 expression is regulated independently. This idea is consistent with the fact that cuc1-1 and cuc2 single mutants develop a normal embryonic SAM (Aida et al., 1997).

Adventitious shoot formation in 35S::CUC1

Ectopic shoot formation in 35S::CUC1 plants suggests that CUC1 can strongly promote SAM initiation. There are several
interesting points about the ectopic shoot formation in 35S::CUC1 plants.

In 35S::CUC1 lines, adventitious shoots are formed mainly on cotyledons, rarely on rosette leaves and not on cauline leaves. In contrast, in 35S::KNAT1 plants, ectopic meristems are formed on rosette and cauline leaves and not on cotyledons (Lincoln et al., 1994; Chuck et al., 1996). This difference may reflect the developmental phase when each gene could function in normal development; transcripts of KNAT1 are not detected in the presumptive SAM during embryogenesis (Lincoln et al., 1994), while CUC1 mRNA was only weakly detected in the vegetative meristems. Cells in cotyledons may be able to specifically respond to genes expressed during embryogenesis, while cells in rosette and cauline leaves may easily respond to genes expressed in postembryonic development. For example, the maize KNOTTED1 gene is expressed both in the presumptive embryonic SAM during embryogenesis and in the postembryonic SAMs, and overexpression of this gene in tobacco causes ectopic shoot formation on cotyledons as well as on leaves (Sinha et al., 1993; Smith et al., 1995).

The ectopic meristems in 35S::CUC1 were always formed on the adaxial surface but not on the abaxial surface of cotyledons and leaves. This phenomenon is also observed in transformants ectopically expressing the KNOTTED1, KNAT1 or cytokinin-synthesizing gene (Estruch et al., 1991; Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996). These results may reflect a difference between the adaxial and abaxial sides of cotyledons and leaves. It is hypothesized that the adaxial side of cotyledons and leaves has an important role in promoting the development of the SAMs. This idea is supported by the following four lines of evidence. (1) An Arabidopsis mutant, phabulosa-1d, in which both sides of leaves develop with adaxial characteristics, shows ectopic SAM formation from the underside of the leaves (McCouch and Barton, 1998). (2) The ZWILLE/PINHEAD (ZLL/PNH) gene, which is required for the development of the embryonic and axillary SAMs, is expressed in the adaxial half of cotyledons and leaves but not in the abaxial one (McCouch et al., 1995; Moussian et al., 1998; Lynn et al., 1999). (3) In an Antirrhinum mutant, phantastica, abaxialized leaves are observed, and the SAM does not function properly (Waite and Hudson, 1995; Waite et al., 1998). (4) In transgenic plants ectopically expressing members of the YABBY gene family, abaxialized cotyledons and leaves are observed, and the embryonic and axillary SAMs often fail to develop, while ectopic meristems are sometimes formed in yabby1 yabby3 double mutants (Sawa et al., 1999; Siegfried et al., 1999).

In 35S::CUC1, ectopic shoots were formed near vascular strands of cotyledons. This is the same as in the case of overexpression of the KNOTTED1, KNAT1 or cytokinin-synthesizing genes. This may be due to the strong activity of the 35S promoter in vascular tissues (Jefferson et al., 1987; Schneider et al., 1990). It is also possible that unknown SAM-inducing components exist specifically in vascular tissues. In the latter case, ZLL/PNH may be a promoter of SAM formation, because it is expressed strongly in vascular tissues of cotyledons and leaves (Moussian et al., 1998; Lynn et al., 1999).

**CUC1 is a positive regulator of STM expression**

CUC1, CUC2 and STM are shown to be expressed in overlapping regions in globular-stage embryos (this study; Long et al., 1996; Long and Barton, 1998; Aida et al., 1999). Expression analyses indicate that CUC1 and CUC2 function upstream of STM and are required for the expression of STM; STM is not expressed in cuc1-1 cuc2 double mutant embryos, whereas CUC1 and CUC2 are expressed in stm-1 mutant background (this study; Aida et al., 1999). Moreover, the fact that STM is ectopically expressed in 35S::CUC1 cotyledons strongly suggests that overexpression of CUC1 is sufficient to induce ectopic STM expression. Taken together, these results suggest that CUC1 is a positive regulator of STM expression. However, it remains to be determined how CUC genes regulate STM expression.

**Overexpression of CUC1 leads to the formation of lobed cotyledons**

In 35S::CUC1 transformants, epidermal cells in the sinus-like regions of the lobed cotyledons were small and round. In addition, specialized structures such as stomata and pavement cells were not observed. These observations suggest that these epidermal cells were in an immature state and did not expand or differentiate properly.

The development of these undifferentiated cells may be related with the formation of the lobed cotyledons. In 35S::KNAT1 plants, lobed leaf development is thought to be a result of the exaggeration of serrations by the restriction of growth, especially in sinus regions. Sinuses of wild-type leaves are produced later in leaf development and are relatively undifferentiated, which leads to the hypothesis that sinus cells can easily respond to KNAT1 expression (Chuck et al., 1996). By analogy, wild-type cotyledon primordia may contain relatively undifferentiated regions which are comparable to the sinuses of leaf serrations (Fig. 7A). Overexpression of CUC1 would make these regions more meristematic, inhibit the growth of cells within and change the morphology of cotyledons into lobed structures (Fig. 7B). It is likely that overexpression of CUC1 promotes SAM formation and inhibits the growth of cotyledons by maintaining undifferentiated cells...
in the cotyledons. Therefore it is suggested that CUC1 may function to maintain undifferentiated cells during SAM formation in wild-type plants.

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