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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 overexpressor 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 (Souer 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 (Souer 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 *AP3* (Sablowski and Meyerowitz, 1998). However, involvement of *NAC* genes in 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 allelism tests

cuc2 seeds (ecotype Landsberg *erecta* [L*er*]; Aida et al., 1997) were mutagenized by incubating in 0.4% EMS for 8 hours. Individual M_1 plants were grown and harvested independently. Independent 2200 M_2 lines were screened for seedlings with the cup-shaped cotyledon phenotype. To determine allelism, *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*/+ plants (ecotype L*er*) were crossed to wild-type plants (ecotype Col), and *cuc1-1*/+ *cuc2*/+ plants (F_1) were obtained. Among the progenies of the F_1 plants, 972 of *cuc1-1*/*cuc1-1 cuc2*/*cuc2* double mutant seedlings were examined for recombinations between the *CUC1* locus and PCR markers in chromosome 3. Degenerate primers used to amplify *NAC* genes from the YAC clone were PPGFRFHPf (5′-ccicciggitt[c/t][a/c]gitt[c/t]ca[c/t]cc-3′ [i; inosine]) and WVMHEYRLr (5′-ia[a/g]ic[g/t][a/g]ta[c/t]tc[a/g]tg-catiaccc-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 *Hin*dIII-*Xho*I 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′-aaaagcttcttcttgtgccgacaatgg-3′) and P4-5 (5′-gtctgcagaccgcatggcga-tcagagag-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′-aatcacttctcgggatgtcg-3′) and AtNAC1-5 (5′-atttgccgtcaaggcgatac-3′). Endogenous *CUC1* DNAs were obtained by PCR with primers AtNAC1-2 (5′-taggcttagtggagacactg-3′) and P4-17 (5′-acaacctctccaagcaagtc-3′; located outside the 3.8-kb region). These PCR products were reamplified with the dCAPS primers, and amplified fragments were digested with *Hin*cII, which produces a 105-bp fragment for the L*er* allele and 86- and 19-bp fragments for the *cuc1-1* allele. The *cuc2* mutation was detected with primers CUP1B-1 (5′-cggaggctaaagaagtacca-3′) and Tag1-4 (5′-ctcgagagattgagtcgcc-gtttg-3′), while the L*er* allele of *CUC2* was detected with primers CUP1B-1 and CUP1B-4 (5′-atccacattattaccacgccc-3′).

Sequence analyses and database searches

Phylogenetic trees and alignments were constructed by using Genetyx 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 in a 20 μl reaction. Parts of the reactions were used as a template for PCR. The PCR programs were as follows: 0.5 minutes at 92°C, 1 minute at 55°C and 2 minutes at 72°C was performed 15 or 16 cycles for *CUC1* and *CUC2*, 18 cycles for *STM* and 10 or 11 cycles for *ACT8*. These cycles, in which PCR products were in the exponential increase, were determined by testing 6-20 cycles. Amplified fragments were separated on agarose gel, blotted and detected by Southern hybridization probed with the corresponding cDNA fragments. Southern hybridization was performed by using ECL direct nucleic acid labeling and detection systems (Amersham). Signals on the X-ray film were quantitated by using NIH image program. The primers used for amplification were: CUP3 (5′-cagccaatatcttccaccggg-3′) and CUP11 (5′-ggagaggtgggagtgagacgga-3′) for *CUC2*; P4-6 (5′-tctgagccttgggagcttcc-3′) and P4-5 for *CUC1*; STMHf (5′-gcccatcatgacatcacatc-3′) and STMHr (5′-ctggatcttcacc-aagacac-3′) for *STM* and ACT8f (5′-atgaagattaaggtcgtggca-3′) and ACT8r (5′-tccgagtttgaagaggctac-3′) for *ACT8*. *ACT8* was used as an internal control (An et al., 1996). In the case of RT-PCR in the L*er*, *cuc1-1* or *cuc2* mutant background (Fig. 2B), 0.65 μg of DNase I-

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 456-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 (L*er* ecotype) plants were transformed using the vacuum infiltration method (Bechtold et al., 1993) with *Agrobacterium* strain MP90. Transgenic plants (T_1) were screened on agar plates containing kanamycin (50 μg/ml). For RT-PCR analyses, RNAs were extracted from next progenies (T_2) of each T_1 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*.

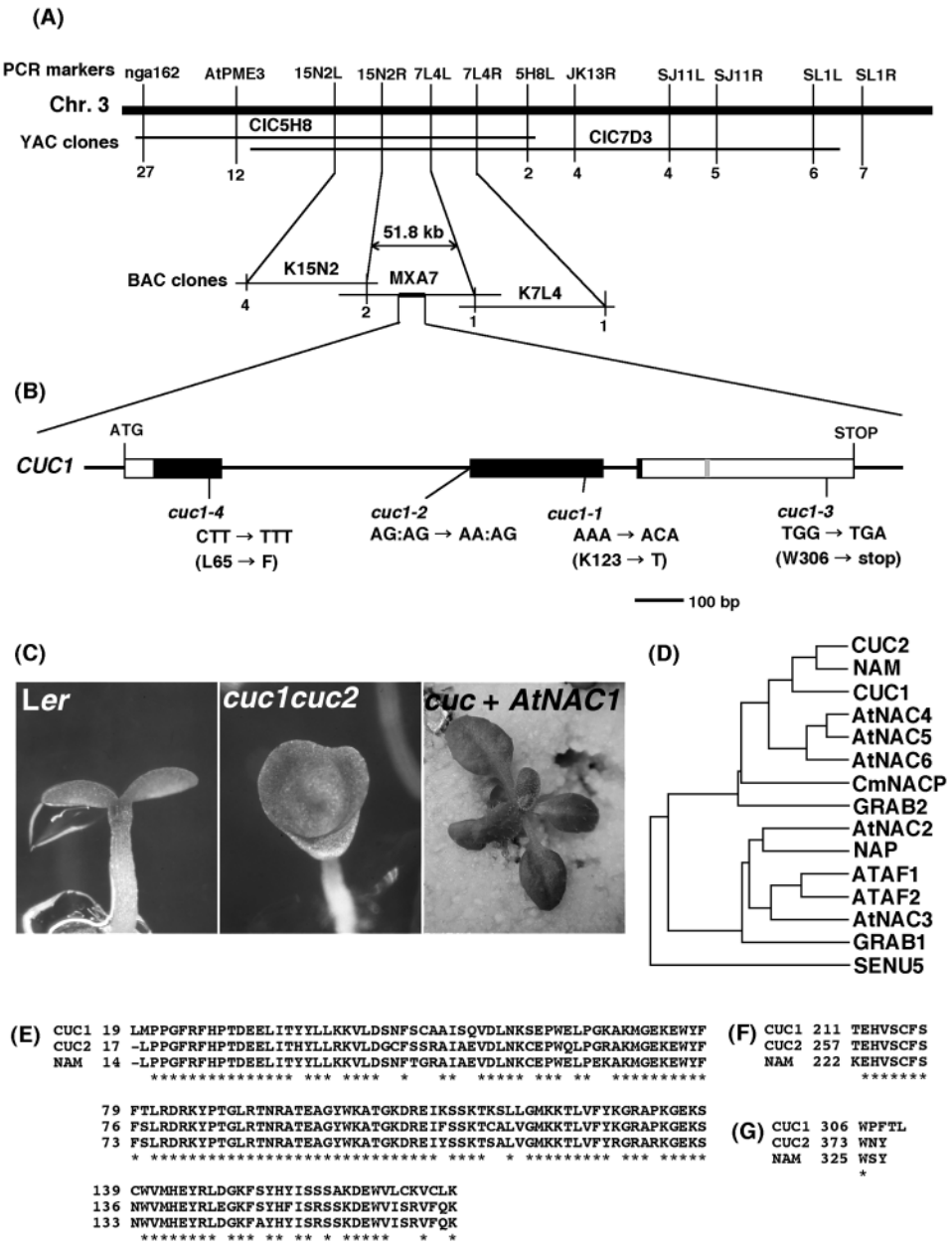

Fig. 1. Map-based cloning and the structure of *CUC1*. *(A)* Physical mapping of the *CUC1* locus. The number of recombination events is given below the PCR markers. (B) Structure of the *CUC1* gene. Boxes represent the open reading frame, and lines indicate introns or untranslated regions. Mutations in four *cuc1* alleles are shown below. Filled box, NAC box; shaded box, conserved sequence in the 3′-terminal half. (C) Left, L*er* seedling; middle, *cuc1-1 cuc2* seedling; right, *cuc1-1 cuc2* plant transformed with the *AtNAC1* genome fragment. (D) The phylogenic tree of deduced amino acid sequences of the NAC domains of *Arabidopsis* CUC1, CUC2 (Aida et al., 1997), AtNAC2-AtNAC6, ATAF1 (accession number: X74756), ATAF2 (accession number: X74755), and NAP (Sablowski and Meyerowitz, 1998), petunia NAM (Souer et al., 1996), tomato SENU5 (John et al., 1997), wheat GRAB1 and GRAB2 (Xie et al., 1999), and pumpkin CmNACP (Ruiz-Medrano et al., 1999) constructed by using the unweighted pair group method (Michener and Sokal, 1957). (E) Alignment of the NAC domains of CUC1, CUC2 and NAM. (F) Alignment of the conserved regions in the C-terminal halves of CUC1, CUC2 and NAM. (G) Alignment of the amino acid sequences near the C-terminal ends of CUC1, CUC2 and NAM proteins. In E-G, amino acids that are identical in CUC1, CUC2 and NAM proteins are marked by asterisks.

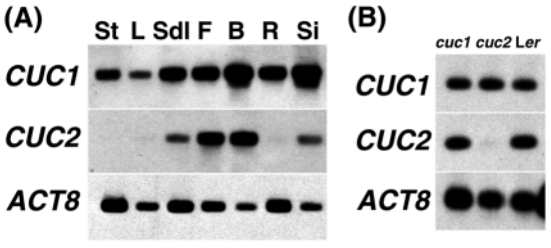

Fig. 2. RT-PCR analyses of the expression of the *CUC* genes. (A) Expression of *CUC1* and *CUC2* RNA in various tissues of L*er*. St, inflorescence stems; L, rosette leaves; Sdl, aerial parts of 7-day-old seedlings; F, flowers; B, floral buds; R, roots; Si, siliques. (B) Expression of *CUC1* and *CUC2* RNA in *cuc1-1* and *cuc2* floral buds. In (A) and (B), *ACT8* was used as an internal control (An et al., 1996).

To examine the spatial and temporal expression of the *CUC1* gene in wild-type development, we performed in situ hybridization experiments. In wild-type embryos, *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, *CUC1* mRNA was detected between the two cotyledon primordia (Fig. 3B, arrowhead); *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, *CUC1* expression extended below the O′ line (Fig. 3C; West and Harada, 1993). These expression patterns of *CUC1* overlapped with those of *CUC2* and *STM* (Long et al., 1996; Aida et al., 1999). In late-

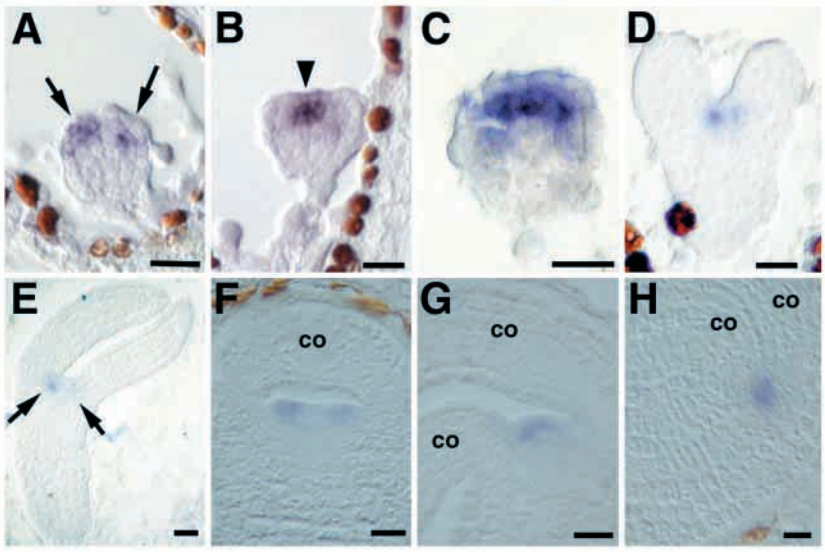

Fig. 3. Localization of *CUC1* mRNA in embryos. (A) Sagittal longitudinal section through a globular-stage embryo. Arrows indicate *CUC1* expression in two separate regions. (B) Frontal longitudinal section through an early-heart-stage embryo. The arrowhead indicates the boundary between cotyledon primordia. (C) Sagittal longitudinal section through an early-heart-stage embryo. (D) Frontal longitudinal section through a late-heart-stage embryo. (E) Frontal longitudinal section through a bending-cotyledon-stage embryo. Arrows indicate the boundaries between cotyledon primordia and the SAM. (F) Sagittal longitudinal section through a mature-stage embryo. (G) Frontal longitudinal section through a mature-stage embryo. (H) Frontal longitudinal section through a mature-stage embryo of *stm-1*. co, cotyledon. The definition of section planes in embryo development was according to Long and Barton (1998). Scale bars, 20 μm.

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-

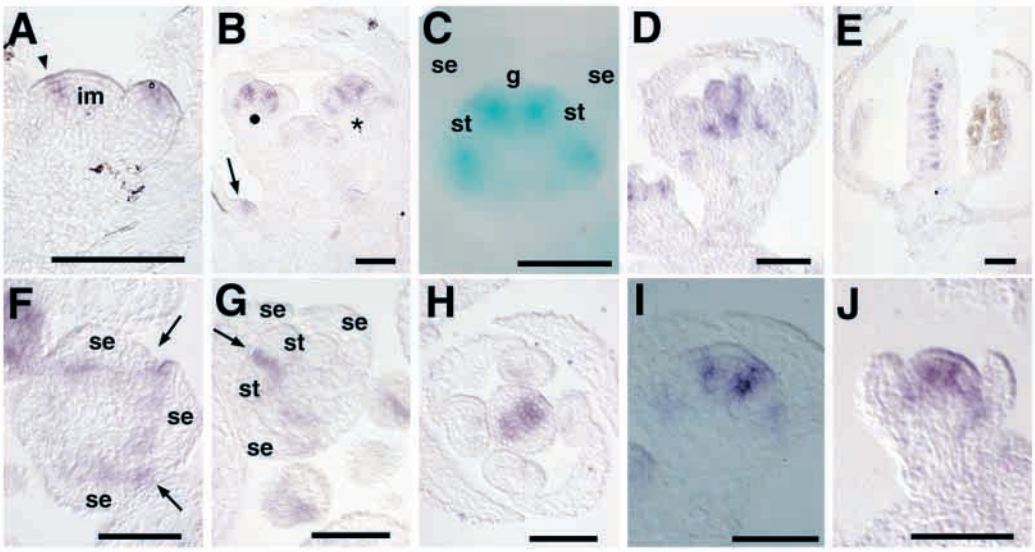

Fig. 4. *CUC1* expression in inflorescences. (A) Longitudinal section through the inflorescence meristem. Arrowhead indicates boundary region between the inflorescence meristem (im) and a stage 1 floral meristem. (B) Longitudinal section through an inflorescence shoot. Filled circle, stage 4 flower; asterisk, stage 6 flower; arrow, axillary meristem. (C) Side view of a stage 5 flower showing CUC1:GUS expression. (D) Longitudinal section through a stage 7 flower. (E) Longitudinal section through a stage 10-11 flower. (F) Transverse section through a stage 4 floral meristem. Arrows indicate boundaries between individual sepal primordia. (G) Oblique transverse section through a stage 5-6 floral meristem. Arrow indicates the boundary between stamen primordia. (H) Transverse section through a stage 7 flower. (I) Longitudinal section through a stage 5 *cuc1-1* flower. (J) Longitudinal section through a stage 4 *cuc2* flower. The stage of flower development was determined as described by Smyth et al. (1990). se, sepal; st, stamen primordia; g, gynoecium. Scale bars, 50 μm.

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. 4I,J). 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 (T_1) showed fused cotyledons and fused floral organs (sepals and stamens) in the next T_2 generation (data not shown). In these lines, expression levels of *CUC1* were reduced by co-suppression (data not shown).

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

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

Two-week-old plants were scored for the above phenotypes.

The remaining 14 kanamycin-resistant transformants showed a novel phenotype in the T_2 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

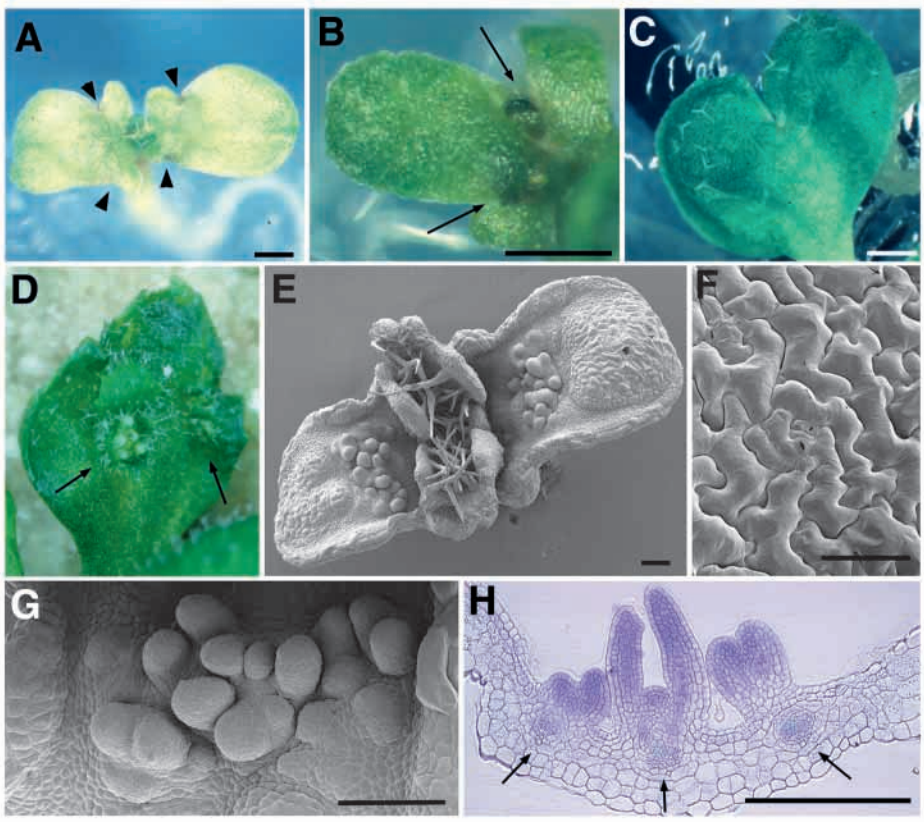

Fig. 5. Phenotypes of 35S::*CUC1*. (A) 7-day-old 35S::*CUC1* seedling. Arrowheads indicate sinuses. (B) Lobed cotyledon of a 35S::*CUC1* seedling. Arrows indicate adventitious shoots. (C) Fused leaf of a 35S::*CUC1*. (D) A 35S::*CUC1* leaf. Arrows indicate adventitious inflorescences. (E) SEM image of a 35S::*CUC1* seedling. (F) Adaxial epidermal cells of a wild-type cotyledon. (G) Adaxial surface of the 35S::*CUC1* cotyledon in E. (H) Transverse section of a 35S::*CUC1* cotyledon. Arrows indicate vascular strands. Scale bars, 1 mm in A-C, H; 100 μm in E-G.

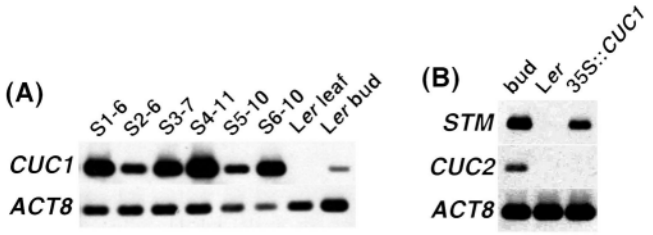

Fig. 6. Expression analyses in 35S::*CUC1*. (A) Quantitative RT-PCR analysis of *CUC1* expression in 35S::*CUC1* lines. RNAs were isolated from rosette leaves of 35S::*CUC1* (S1-6, S2-6, S3-7, S4-11, S5-10 and S6-10) and L*er* (L*er* leaf), and from floral buds of L*er* (L*er* bud). (B) Quantitative RT-PCR analysis of *CUC2* and *STM* expression in 35S::*CUC1* cotyledons (35S::*CUC1*), L*er* cotyledons (L*er*) and L*er* 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 (Fig. 5D). All six 35S::*CUC1* plants produced inflorescences, flowers and viable seeds from the primary SAM (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 cotyledonary 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 (McConnell 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 (McConnell 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 (Waites and Hudson, 1995; Waites 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.

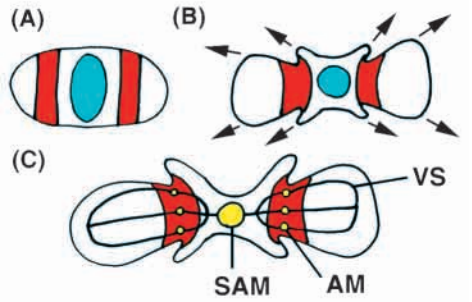

Fig. 7. Model for the abnormal cotyledon development in 35S::*CUC1* plants as viewed from above. (A) Apical region of an embryo. Red regions indicate hypothetical zones that are highly responsive to the expression of *CUC1*. Blue region, presumptive SAM. (B) In the red regions meristematic cells are observed, and cell expansion is restricted. As a result, lobed cotyledons are formed. Arrows indicate the direction of the growth. (C) In the cotyledons, adventitious meristems (AMs) are formed on the adaxial surface within the meristematic regions. Adventitious meristems are formed near vascular strands (VSs) of the cotyledons. Yellow regions, meristems.

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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