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Laboratoire de Biologie Cellulaire, Institut J. P. Bourgin, INRA, 78026 Versailles Cedex, France
*Author for correspondence (e-mail: laufs@versailles.inra.fr)

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Summary

We have analysed the role of a microRNA, *miR164*, in boundary formation during organ initiation from *Arabidopsis* meristems. The establishment and maintenance of the boundary domain are controlled by three partially redundant genes, *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2* and *CUC3*. We show that *miR164* overexpression phenocopies the *cuc1 cuc2* double mutant by inducing post-transcriptional downregulation of *CUC1* and *CUC2* but not *CUC3* mRNA levels. Disruption of *CUC2* regulation by *miR164*, either by making *CUC2* resistant to the miRNA or by reducing miRNA levels leads to similar enlarged boundary domains. We relate this enlargement to the division patterns of the boundary cells. We propose that *miR164* constrains the expansion of the boundary domain, by degrading *CUC1* and *CUC2* mRNAs.

Supplemental data available online

Key words: MicroRNA, Meristem, Boundary, Cell fate, Differentiation, Proliferation

Introduction

During pattern formation in plants and animals, groups of cells are divided into domains that acquire different developmental fates. This process requires the establishment of precise gene expression patterns that are maintained despite continuous growth and cell division. A recently discovered class of small RNAs, the microRNAs (miRNAs) involved in gene expression regulation may contribute to this. In particular, plant miRNAs have been proposed to remove the transcripts of important regulators in some daughter cell lineages and thus could participate in the stabilisation of gene expression patterns (Rhoades et al., 2002). To further test this hypothesis, we analysed the role of one miRNA, *miR164*, during the process of boundary formation around organ primordia in *Arabidopsis* meristems.

MiRNAs are small, single-stranded RNAs of about 21 nucleotides found in both animals and plants that post-transcriptionally regulate gene expression (for reviews, see Bartel, 2004; Lai, 2003). Animal miRNAs are transcribed as long primary transcripts (pri-miRNAs) that are first processed into hairpin precursors of about 70 nucleotides (pre-miRNAs) and then into mature miRNAs. Although the cleavage affects both strands of the hairpin precursors, only one strand, the mature miRNA, is preferentially accumulated and incorporated into a ribonucleoprotein complex, the miRNP complex (Khvorova et al., 2003; Mourelatos et al., 2002; Schwarz et al., 2003). Interaction of the miRNA with imperfect complementary sequences located in the 3′ untranslated region (UTR) of the target mRNAs leads to translational attenuation. Conversely, plant miRNAs are perfectly or almost perfectly complementary to their targets (Rhoades et al., 2002), and their interaction triggers the cleavage of the mRNA (Han et al., 2004; Kasschau et al., 2003; Llave et al., 2002; Palatnik et al., 2003; Tang et al., 2003; Vazquez et al., 2004; Xie et al., 2003), although examples of translational attenuation have also been reported (Aukerman and Sakai, 2003; Chen, 2004).

In animals, most evidence for miRNA regulation of gene expression results from classical genetic approaches, although potential targets of miRNAs have been recently predicted by bioinformatics (Enright et al., 2003; Lewis et al., 2003; Rajewsky and Socci, 2004). miRNAs were first identified as regulators of the developmental timing in *C. elegans* (Abrahante et al., 2003; Lin et al., 2003; Reinhart et al., 2000; Slack et al., 2000). Additional evidence suggests that they may be involved in spatial patterning processes. For example, left-right asymmetry during neuronal patterning in *C. elegans* is controlled by a miRNA (Johnston and Hobert, 2003). In *Drosophila*, the *Bantam* miRNA, the expression of which responds to patterning cues, promotes cell proliferation and prevents apoptosis by targeting the pro-apoptotic gene *hid* (Brennecke et al., 2003). Thus, *Bantam* may participate in the coordination between patterning events and downstream control of cell death and cell proliferation.

In plants, most of the miRNA targets predicted by bioinformatics are transcription factors involved in the control of development, raising the possibility that miRNAs may play an important role in this process (Rhoades et al., 2002). Organogenesis in plants, in contrast to animals, proceeds throughout their life span as new tissues and organs are continuously produced by meristems. For example, the shoot apical meristem and a related structure, the floral meristem, initiate primordia of lateral organs such as leaves, sepals or stamens. A family of miRNA, *miR172* negatively regulates *APETALA2*-like transcription factors, thus controlling flowering time and floral organ identity (Aukerman and Sakai, 2003; Chen, 2004). Another miRNA family, *JAW/miR159*, which

negatively regulates several members of the TCP and MYB transcription factor families is involved in leaf development (Palatnik et al., 2003). miRNAs have also a central role in lateral organ polarisation. Lateral organ polarity is controlled by three members of a homeodomain/leucine zipper transcription factor family, *PHABULOSA* (*PHB*), *PHAVULOTA* (*PHV*) and *REVOLUTA* (*REV*) (Emery et al., 2003; McConnell et al., 2001; Otsuga et al., 2001). These genes and two evolutionary conserved miRNA, *miR165* and *miR166* predicted to target them, are expressed in complementary domains of the developing lateral organs (Juarez et al., 2004; Kidner and Martienssen, 2004). miRNA-resistant forms of these targets are ectopically expressed in the developing primordia, suggesting that miRNAs normally limit their expression pattern (Emery et al., 2003; Juarez et al., 2004; Kidner and Martienssen, 2004; McConnell et al., 2001; Tang et al., 2003).

Here we have analysed the role of a miRNA, *miR164*, in the regulation of the boundary domain around developing primordia at the shoot apical and floral meristems. Boundary establishment and maintenance is controlled in *Arabidopsis* by three partially redundant genes, *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2* and *CUC3* (Aida et al., 1997; Aida et al., 1999; Takada et al., 2001; Vroemen et al., 2003). These three members of the NAC transcription factor family are expressed in the cells forming the boundary domain around primordia where they may repress growth (Aida et al., 1999). A single mutation of either CUC gene has no major effect on boundary formation, whereas double mutants have fused cotyledons reflecting abnormal boundary specification during embryo development (Aida et al., 1997; Vroemen et al., 2003). Later on, the *cuc1 cuc2* double mutant phenotype is restricted to the flowers that form partially fused organs (Aida et al., 1997). The absence of a mutant phenotype during the vegetative phase and in the inflorescence stem was proposed to be due to partial redundancy between the three CUC genes identified in *Arabidopsis*. In addition to their role in boundary specification, the CUC genes are also involved in meristem establishment during embryogenesis. Indeed, the CUC genes promote the expression of the *SHOOT MERISTEMLESS* (*STM*) gene, a central determinant of meristem identity (Daimon et al., 2003; Hibara et al., 2003; Takada et al., 2001; Vroemen et al., 2003). Sequence homology suggests that *CUC1* and *CUC2* mRNAs could be targeted by *miR164* (Rhoades et al., 2002). Accordingly, their expression levels are increased in *Arabidopsis* backgrounds with an impaired miRNA pathway (Kasschau et al., 2003; Vazquez et al., 2004). We show that *miR164* targets *CUC1* and *CUC2* but not *CUC3* mRNAs for degradation, in planta. Disruption of *CUC2* regulation by the *miR164*, either by making it resistant to the miRNA or by reducing the miRNA level leads to a similar boundary enlargement phenotype. We traced this modification back to the proliferative activity of the boundary cells. Therefore, we propose a model where *miR164* mediates the degradation of *CUC1* and *CUC2* mRNAs, and thus limits the expansion of the boundary domain.

Materials and methods

Constructs

For the *2x35S::miR164A* and *2x35S::miR164B* constructs, 1049 bp and 1021 bp fragments of genomic DNA centred on *MIR164A* and *MIR164B* precursors, respectively (Reinhart et al., 2002) were amplified by PCR using primer pairs miR164A-2/miR164A-3 and miR164B-1/miR164B-2, respectively, and cloned between the double *35S* promoter and the *35S* terminator from *Cauliflower virus* of plasmid pLBR19 in the binary vector pGreen0029 (Hellens et al., 2000). For the *2x35S::erGFP*, the *erGFP* was excised from pMCB56 (Fernandez-Abalos et al., 1998) and further cloned as for the *miR164*. Mutations were introduced by PCR into the *CUC2* cDNA obtained from REGIA consortium. Mutated or wild-type *CUC2s* were introduced between the *alcA* promoter and *Cauliflower virus 35S* terminator of pL4 plasmid and the *alcA::CUC2s* expression cassettes were introduced into the pEC2 binary vector. The wild-type *CUC2* and *CUC2-m4* were fused to a HA-tag and inserted between the double *35S* promoter and the *35S* terminator from *Cauliflower virus* of plasmid pLBR19 in the binary vector pGreen0029 (Hellens et al., 2000). The *STM::ALCR alcA::GUS* and *STM::ALCR alcA::erGFP* constructs were made following the same strategy as described by Deveaux et al. (Deveaux et al., 2003): 4.4 kb of *STM* regulatory sequences ending 13 bp before the ATG were PCR-amplified using STM-1 and STM-2 and cloned into pLP999 or pLP962. pGreen-based binary vectors were electroporated into *Agrobacterium tumefaciens* strain GV3101 together with the pSoup plasmid (Hellens et al., 2000), whereas strain C58 was used for pEC2-derived vectors.

Plant material

Plants were transformed by floral-dip (Clough and Bent, 1998). *AlcA::CUC2 STM::ALCR alcA::erGFP* lines were generated by retransforming a *STM::ALCR alcA::erGFP* line. The *LFY::ALCR alcA::GUS* and *LFY::ALCR alcA::GUS alcAH4GFP* have been described previously (Deveaux et al., 2003). The M0223 enhancer trap line described by Cary et al. (Cary et al., 2002) comes from the Hasselhoff collection and was provided by the Nottingham Arabidopsis Biological Stock Centre. The *hyl1-1* (Lu and Fedoroff, 2000) and *hen1-5* (Vazquez et al., 2004) mutants were kindly provided by N. Fedoroff and H. Vaucheret, respectively, and the *dcl1-9* mutant was provided by the NASC.

Plant growth in vitro or in the greenhouse, and ethanol induction in the greenhouse have been described before (Deveaux et al., 2003) with the exception that 75% ethanol was used for vapour induction instead of 95%. For in vitro induction, 0.1% of ethanol was added to the growth media before pouring the plates.

RNA analysis

Total RNA was extracted from inflorescence apices using TRIZOL (Invitrogen) according to manufacturer's instructions.

For miRNA detection, 30 μg of total RNA were separated overnight on a 15% acrylamide, 8 M urea gel and blotted on Hybond-NX membranes using a BioRad semi-dry blotter. Filters were hybridised overnight in Church buffer at 30°C with end-labelled primers, then washed for 1 hour in 2×SSC, 0.1% SDS. Blots were reprobed with a *5S* RNA probe.

For HMW RNA, 20 μg of total RNA were separated on a 1.5% agarose gel, blotted on nylon membranes and probed with a randomly ^{32}P-labelled DNA fragment specific for CUC2 (from 415 bp after the ATG to the STOP codon).

RT-PCR was carried out as previously described (Laufs et al., 2003) using primers located on two different exons to discriminate between genomic contamination and RT products. Furthermore, the two primers, located on each side of the predicted *miR164* cleavage site, selectively amplified only the uncleaved mRNA. Twenty-three PCR cycles were run for *CUC1*; 21 cycles for *NAC1* and *At5g07680*; 19 cycles for *At5g61430*, *CUC2* and *CUC3*; and 15 cycles for APT. The primers used are indicated in Table 1.

Microscopy and images analyses

Confocal microscopy and image analysis were carried out as described by Deveaux et al. (Deveaux et al., 2003). Scanning electron

Table 1. Primers used in this study

Primer name	Sequence 5′→3′
miR164A-2	TCAATGCGTTACATATGCTG
miR164A-3	CCATGCCATAGAGTAGATGC
miR164B-1	TTTTTGGGTAGCATGTTCAT
miR164B-2	CGCTAACCGAAACTATGTTC
STM-1	GTATAATTTGATAAATATTCACTTTGTGTTTCGTC
STM-2	TCACTAGTATTATTATTCACTTTGGCTTTGCTATA
CUC1-RT1	AACGCCACGCCATCACCGAC
CUC1-RT2	TGCATGAGTATCGCCTTGAC
CUC2-RT1	AGGCCGTAGTAGTAGTAGGG
CUC2-RT2	TGAAGGCAAATTCTCTTACC
CUC3-RT1	GAGAGACGACAGGGTTGATT
CUC3-RT2	TGGCCTCAAGACTAAGTGG
At5g61430-RT1	AGAACCGGGCTCTGTAGATT
At5g61430-RT2	TTCTCTGCCCATAACTTGCCG
At5g07680-RT1	GATTGGAACTCTCGGAAATG
At5g07680-RT2	GTGATGCATGAGTATAGGCTAGAT
NAC1-RT1	GGGTTAGGGTTCTTGCATGG
NAC1-RT2	CGAGGCCGTAAAACCGAT
APT-RT1	CCTTTCCCTTAAGCTCTG
APT-RT2	TCCCAGAATCGCTAAGATTGCC

microscopy was carried out according to Bertrand et al. (Bertrand et al., 2003).

Results

miR164 overexpression phenocopies the phenotype of the *cuc1 cuc2* double mutant

In order to analyse the role of *miR164* during shoot apical and floral meristem development, we generated *Arabidopsis* lines overexpressing *miR164*. For this, 1049 bp and 1021 bp of genomic sequences centred on the two loci predicted to code for *miR164* [*MIR164A* and *MIR164B*, respectively (Reinhart et al., 2002)] were cloned under the control of the strong *Cauliflower mosaic* virus double *35S* promoter (*2x35S*, Fig. 1A) and transformed into wild-type Wassilevskija (WS) or Landsberg *erecta* (L*er*) ecotypes. Plants carrying the *2x35S::miR164A* or *2x35S::miR164B* constructs showed similar phenotype modifications and will be called hereafter *2x35S::miR164*. Whereas wild-type or control lines expressing erGFP showed free sepals (Fig. 1B, part 1), sepals of *2x35S::miR164* flowers were fused along their margins (Fig. 1B, parts 2,3). Overexpression of the *MIR164B* locus led generally to stronger defects than the *MIR164A* locus (Fig. 1B, part 5). Fusion of the sepals along their whole margin prevented petal and stamen expansion (Fig. 1B, parts 3,4). No significant reduction of stamen number was observed (not shown) in the transgenic lines, whereas lines showing a high degree of sepal fusion also had fewer petals (Fig. 1B, part 6). Similar sepal fusion and reductions in petal number were reported for flowers of the *cuc1 cuc2* double mutant (Aida et al., 1997). The *cuc1 cuc2* double mutants also show stamen fusion. This did not occur when the *2x35S::miR164* constructs were introduced into WS but could occasionally be observed when they were introduced into L*er* (not shown), the ecotype in which the original *cuc1 cuc2* double mutant was described (Aida et al., 1997).

In addition to the flower phenotype, ~15% of the *2x35S::miR164* primary transformants had embryo patterning defects, such as cup-shaped or partially fused cotyledons (Fig. 1C). The cup-shaped cotyledon phenotype is characteristic for the *cuc1 cuc2* double mutant (Aida et al., 1997), while partial cotyledon fusion is infrequently observed in *cuc1* or *cuc2* single mutants (Aida et al., 1997; Vroemen et al., 2003).

In summary, *2x35S::miR164A* and *2x35S::miR164B* lines exhibited embryo patterning and floral defects that are characteristic for *Arabidopsis* lines with reduced CUC1 and/or CUC2 activity.

Northern blot analysis using a probe complementary to *miR164* revealed a small RNA of ~21-22 nucleotide whose level was increased in the *2x35S::miR164* lines compared with wild-type or *2x35S::erGFP* plants and correlated with the phenotype intensity of the *2x35S::miR164* lines (Fig. 1D, parts 1,2). We next checked if the RNA species we detected corresponded to a single-stranded miRNA or to a double-stranded siRNA. To achieve this, we performed additional northern blots to detect *miR164A** and *miR164B**, the complementary strands to *miR164* that result from RNaseIII processing of *miR164A* and *miR164B* precursors, respectively (Fig. 1D, parts 1,3). The accumulation level of *miR164B** is below detection level in both wild-type and *2x35S::miR164B* plants (Fig. 1D, part 3). Whereas *miR164A** could not be detected in wild-type plants, it accumulated in the *2x35S::miR164A* line, though at a ~10-20 times lower level than *miR164* (Fig. 1D, part 3). A similar low-level of the *miR164A** strand has been reported previously for plant (Reinhart et al., 2002) or animal miRNAs (Lagos-Quintana et al., 2003; Lagos-Quintana et al., 2002; Lau et al., 2001; Lim et al., 2003; Mourelatos et al., 2002). We therefore concluded that bona fide *miR164* accumulated in the *2x35S::miR164* lines.

In conclusion, *miR164* overexpression phenocopied the *cuc1 cuc2* double mutants and the severity of the phenotype correlated with the level of *miR164* accumulation.

miR164 primarily targets four genes of the NAC family

miR164 was predicted by Rhoades et al. (Rhoades et al., 2002) to target 5 members of the NAC gene family: *CUC1* and *CUC2*, *NAC1* (*At1g56010*) that has been implicated in lateral root development (Xie et al., 2000) and two other uncharacterised members (*At5g07680* and *At5g61430*). We analysed by RT-PCR the effects of *miR164* overexpression on the steady state accumulation levels of the five predicted targets and of *CUC3*, a gene partially redundant with *CUC1* and *CUC2* but lacking a *miR164*-binding site.

CUC1 and *CUC2* mRNA levels in *2x35S::miR164* lines were reduced compared with wild-type plants (Fig. 2A). The reduction could reach 90% of the wild-type level and correlated with the intensity of the floral defect phenotype. Although the *CUC3* mRNA accumulation was reduced in the strong lines, the amplitude was lower compared with *CUC1* and *CUC2*. It has been reported that the expression of *CUC3* is abolished or reduced in the absence of both CUC1 and CUC2 activities (Vroemen et al., 2003), showing that CUC1 and CUC2 are redundantly required for *CUC3* expression. Therefore, downregulation of *CUC3* in *miR164* overexpressers is likely to be a secondary effect of *CUC1* and *CUC2* inactivation. The absence of a region complementary to *miR164* in *CUC3* also supports the hypothesis that *CUC3* is not a direct target of *miR164*.

Similar to *CUC1* and *CUC2*, the mRNAs levels of

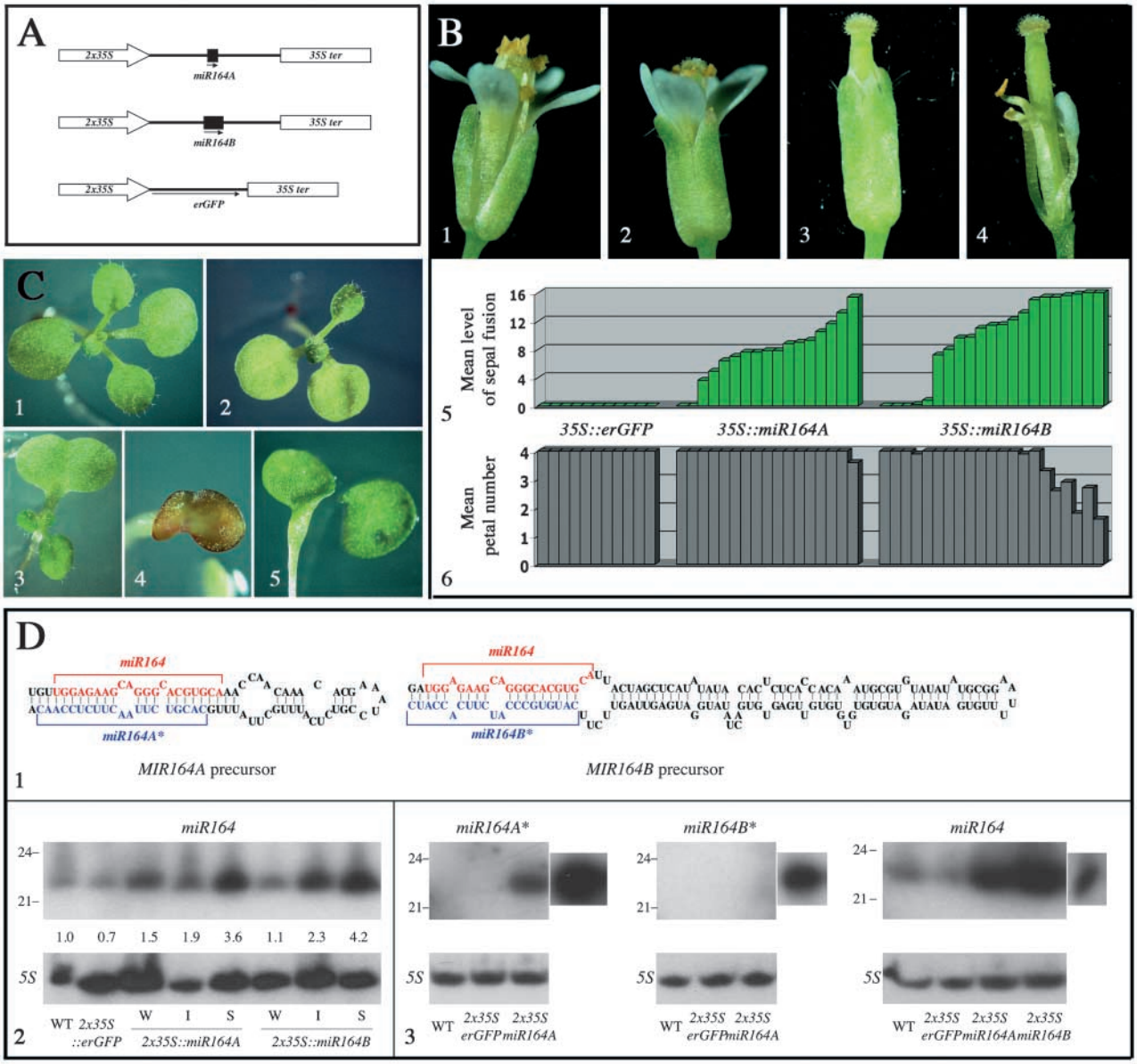

Fig. 1. *miR164* overexpression phenocopies the *cuc1 cuc2* double mutant. (A) The *2x35S::miR164A* and *2x35S::miR164B* constructs contain 1049 bp and 1021 bp of genomic DNA centred on the two predicted pre-miR164 precursors, respectively (black box) (Reinhart et al., 2002) under the control of the *Cauliflower mosaic* virus double *35S* promoter and upstream of the *Cauliflower mosaic* virus *35S* terminator. The *2x35S::erGFP* construct was used as a control. (B) Floral phenotype of wild-type and transgenic lines. (1) Wild-type plants have unfused sepals and expanded petals and stamens. (2,3) *2x35S::miR164A* or *2x35S::miR164B* flowers have fused sepal margins. (3) In lines with strong phenotypes, petals and stamens did not expand. (4) However, dissection of the flower reveals petals and stamens which cannot grow out of the fused sepals, resembling *cuc1 cuc2* double mutant flowers (Aida et al., 1997). The degree of sepal fusion (5) and the petal number (6) was scored in *2x35S:: erGFP*, *2x35S::miR164A* and *2x35S::miR164B* primary transformants. The degree of fusion is expressed on a scale ranging from 0 for normal sepals (as represented in 1) to 16 for the strongest sepal fusion phenotype (as shown in 3). Data represent mean values for 10 flowers per line. (C) Seedling phenotype of wild-type and transgenic lines. (1) Wild-type seedlings have aligned cotyledons. (2-5) *2x35S::miR164A* or *2x35S::miR164B* seedlings have misaligned (2), partially fused cotyledons (3) or cup-shaped cotyledons (4) and petiole fusion revealed by dissection (5). (D) *miR164* is overexpressed in *2x35S::miR164A* and *2x35S::miR164B* lines. **(**1) *MIR164A* and *MIR164B* predicted hairpin precursors (Reinhart et al., 2002). In each precursor, the nucleotides corresponding to *miR164* are red and those corresponding to the other strand resulting from RNase III-mediated cleavage, *miR164A** or *miR164B**, are blue. (2) Detection of *miR164* and control *5S* RNA in apices of wild-type plants, *2x35S::erGFP* and representative *2x35S::miR164A* and *2x35S::miR164B* lines showing either a weak (W), intermediate (I) or strong (S) flower phenotype. The normalised ratio between *miR164* and *5S* RNA expression level is indicated. The migration of 21 and 24 nucleotides DNA primers is indicated. (3) Detection of *miR164A**, *miR164B** and *miR164* and control *5S* RNA, in wild type and in representatives of strong *2x35S::erGFP*, *2x35S::miR164A* and *2x35S::miR164B* lines. Each inset (right) represents the hybridization signal obtained under identical conditions with 100 pg of DNA oligonucleotides corresponding to *miR164A**, *miR164B** and *miR164*.

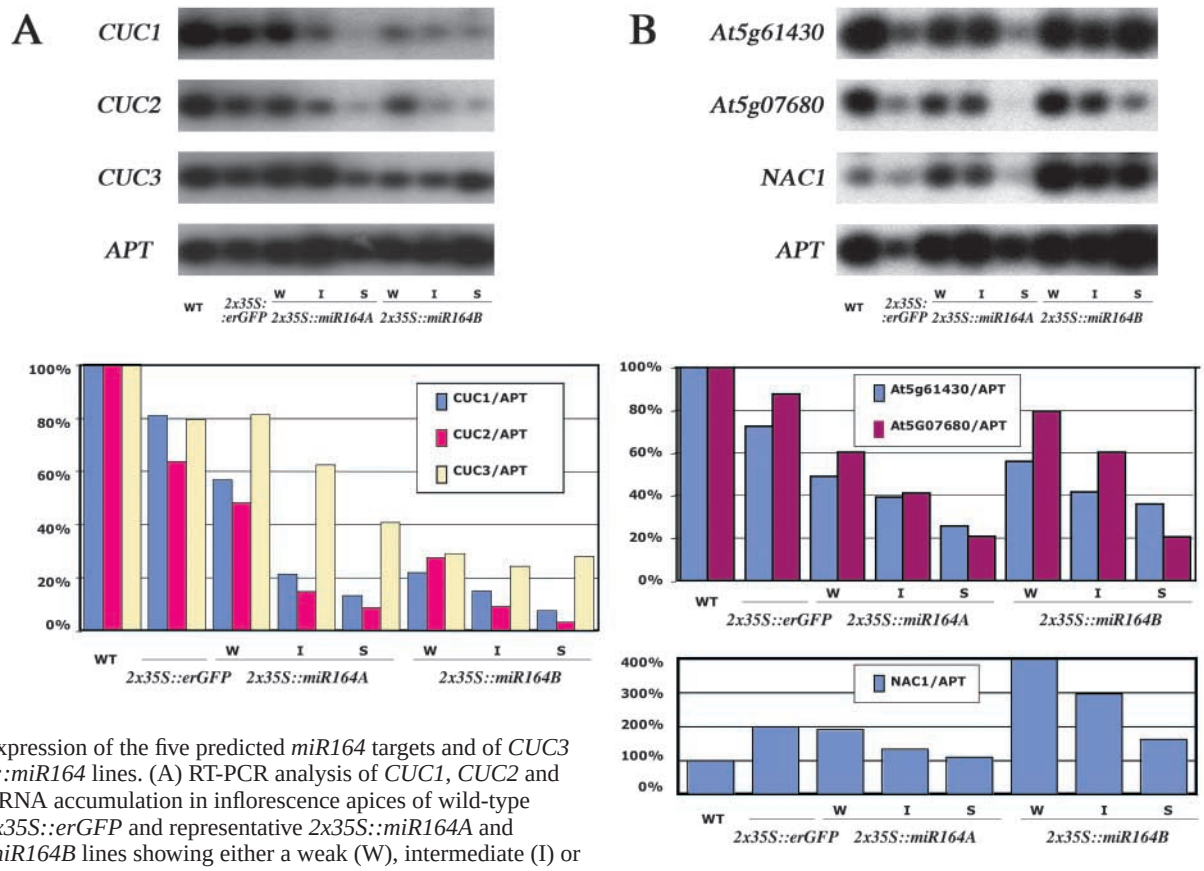

Fig. 2. Expression of the five predicted *miR164* targets and of *CUC3* in *2x35S::miR164* lines. (A) RT-PCR analysis of *CUC1*, *CUC2* and *CUC3* mRNA accumulation in inflorescence apices of wild-type plants, *2x35S::erGFP* and representative *2x35S::miR164A* and *2x35S::miR164B* lines showing either a weak (W), intermediate (I) or strong (S) flower phenotype. *APT* was used as a control. (B) RT-PCR analysis of *At5g61430*, *At5g07680* and *NAC1* mRNA accumulation in inflorescence apices. Histograms show quantification of the target expression level relative to the APT control.

At5g07680 and *At5g61430* were reduced in *miR164* overexpressers, whereas the mRNA level of *NAC1* was not reduced (Fig. 2B). Absence of *NAC1* mRNA downregulation suggests that *miR164* did not interact with it under the conditions we tested or that the interaction did not trigger transcript cleavage but translational attenuation as shown for other plant miRNAs (Aukerman and Sakai, 2003; Chen, 2004).

miR164 regulation of *CUC2* is essential for plant development

In order to assess in planta the importance of *miR164*-guided cleavage of *CUC2*, we modified the *CUC2* mRNA to make it potentially resistant to *miR164*-guided cleavage, without altering the protein sequence. To achieve this, we first introduced four mismatches in the *miR164*-binding site of *CUC2* in addition to the three naturally present (*CUC2-m4* in Fig. 3A) and ubiquitously overexpressed this modified *CUC2* or the wild-type form using the double *35S* promoter in transgenic WS *Arabidopsis* (Fig. 3B). Most of the lines had wrinkled leaves, regardless of the *CUC2* form overexpressed (9/11 *2x35S::CUC2* lines and 7/7 *2x35S::CUC2-m4* lines). Inflorescence size was reduced in six *2x35S::CUC2* lines. A similar phenotype was observed in three *2x35S::CUC2-m4* lines. In addition, two *CUC2-m4* lines showed a more severe phenotype with extreme reduction of internode elongation, small floral organs and reduced fertility. Reduced growth was reported for transgenic lines expressing *CUC1* that, in addition, showed ectopic meristems on the cotyledon surface (Hibara et al., 2003; Takada et al., 2001). We did not observe ectopic meristems, reflecting either different effects of *CUC1* and *CUC2* or specific response of the ecotypes used as CUC1 overexpressers were in L*er* background. The more severe phenotype of the *2x35S::CUC2-m4* lines suggested that *miR164* regulation was important during plant development.

To further investigate the role of *miR164* regulation of *CUC2* during development, we used a strategy that allowed us to induce the expression of different *CUC2* forms (wild-type, m1, m4, c1 and c4 in Fig. 3A) in the boundary domain. To achieve this, we cloned the different *CUC2* forms under the control of the ethanol regulated promoter *alcA* and introduced them into a *STM::ALCR-alcA::erGFP* line. This way, the ethanol-regulated transcription factor ALCR is produced under the control of *SHOOT MERISTEMLESS* (*STM*) regulatory sequences and upon ethanol induction of the plant, binds to the *alcA* promoters and activates simultaneously and in similar domains the expression of *erGFP* and *CUC2* (Fig. 3C). Simultaneous activation of two *alcA* promoters in similar domains has been demonstrated previously (Deveaux et al., 2003). The *STM* regulatory sequences we used are active in the boundary domain of apical and vegetative meristems (see precise description of the expression pattern in Fig. S1 at http://dev.biologists.org/cgi/content/full/131/17/4311/DC1), overlapping with the mRNA accumulation pattern of *CUC2* in this tissues (Ishida et al., 2000). Therefore, the use of *STM* regulatory sequences is an alternative enabling us to temporally

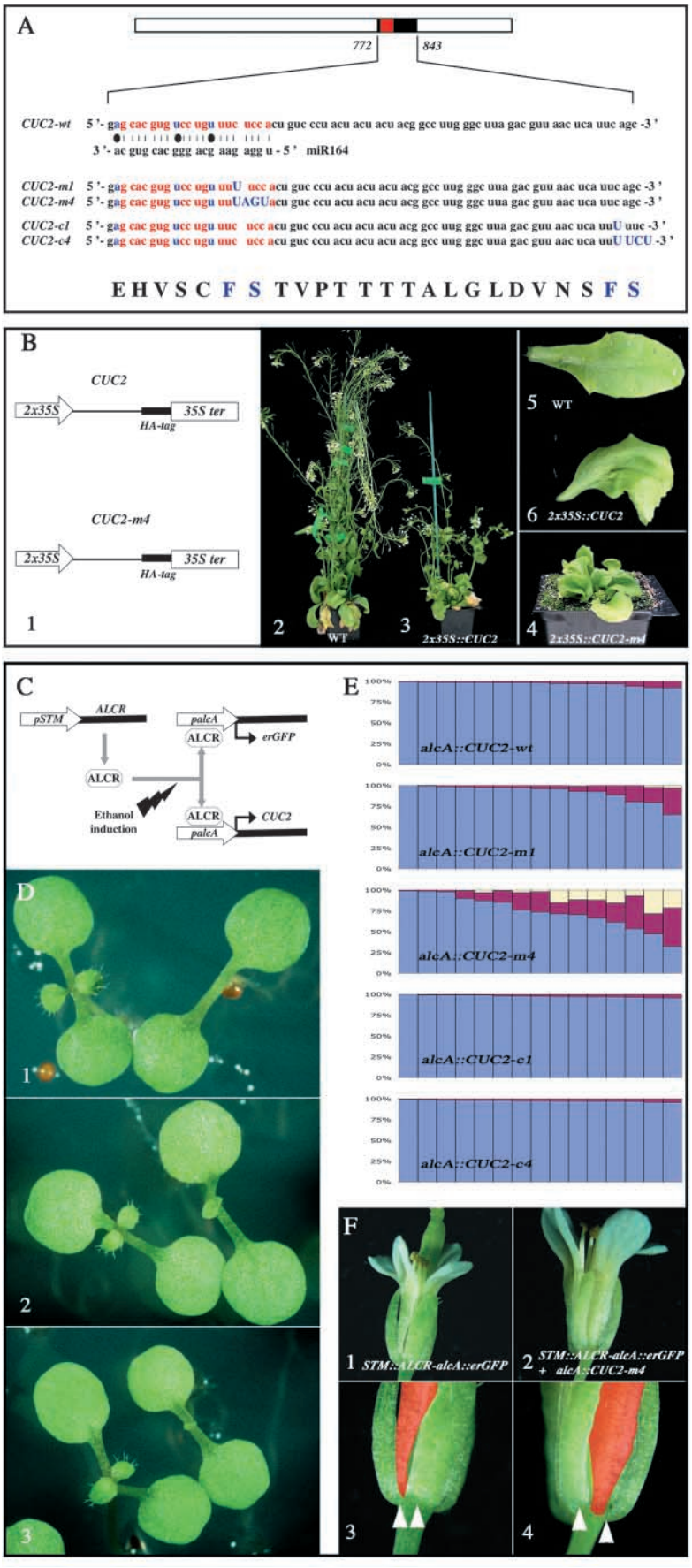

Fig. 3. Importance of *miR164*-mediated regulation of *CUC2* in planta. (A) Partial sequence of *CUC2* mRNA showing the region complementary to *miR164*. Note the three mismatches. One or four additional mismatches were introduced into the binding site of *miR164* in *CUC2* (*CUC2-m1* and *CUC2-m4*). Similar mutations were introduced as controls outside the *miR164*-binding site (*CUC2-c1* and *CUC2-c4*). All the mutations are silent at the protein level. (B) Ubiquitous overexpression of wild-type *CUC2* and *CUC2-m4*. (1) The two *CUC2* forms were cloned under the control of the double *35S* promoter. (2-4) *2x35S::CUC2* and *2x35S::CUC2-m4* transgenic lines show mild and severe growth reduction, respectively, compared with wild type. (4-6) Both *2x35S::CUC2* and *2x35S::CUC2-m4* lines have wrinkled leaves. (C) Strategy used to obtain inducible expression of the wild-type and modified *CUC2s* in the *STM*-expressing domain. The *alcA::CUC2s* constructs were introduced into a *STM::ALCR alcA::erGFP* line. The ALCR transcription factor is expressed under the control of *STM* regulatory sequences and can be activated by ethanol induction. It will then activate simultaneously the expression of the reporter *erGFP* and the different *CUC2s* under the control of the *alcA* promoter. (D) Expression of *miR164*-resistant *CUC2s* leads to abnormal seedling development. No leaf (1, right seedling), asymmetrical leaves (2, right seedling) or small leaves (3, right seedling) were observed in 10-day-old seedlings expressing *miR164*-resistant *CUC2s*, in contrast to what is observed in wild-type plants (left seedling in all panels). (E) Quantification of leaf development in the progeny of 15 transgenic lines expressing the different *alcA::CUC2s*. Ten-day-old seedlings were scored as having normal leaves (blue), abnormal leaves (deep red; such as those shown in D2,3) or no leaf (yellow; such as that shown in D1). At least 100 T2 plants were analysed per line. (F) Phenotype of mature *STM::ALCR-alcA::erGFP* (1,3) or *STM::ALCR-alcA::erGFP alcA::CUC2-m4* (2,4) flowers that have been ethanol-induced for 6 days. In control lines (1,3), the margins of two adjacent sepals (arrowheads) are next to each other, hiding the petal insertion point. In *STM::ALCR-alcA::erGFP alcA::CUC2-m4* lines (2,4), the spacing between the sepals is increased, revealing the insertion point of the petal. Petals are coloured in red in (3) and (4).

control the expression of the *CUC2* genes in the boundary domain.

To analyse in planta the effects of *miR164*-binding site mutation, we ethanol-induced from germination onwards the expression of the different *CUC2* forms in the boundary domain in 15 randomly selected transgenic lines. Seedling development was normal for all lines tested until the formation of the first leaves. Scoring 10-day-old seedlings revealed that the expression of *CUC2-m4* led to severe leaf growth inhibition with absence of any visible leaf in the most extreme case, or two smaller or unequal leaves in the milder cases (Fig. 3D,E). Expression of *CUC2-m1* led also to retarded leaf development though in a smaller proportion of transgenic lines and with a milder effect (Fig. 3D,E). Expression of wild-type *CUC2* or *CUC2* mutated outside the *miR164*-binding

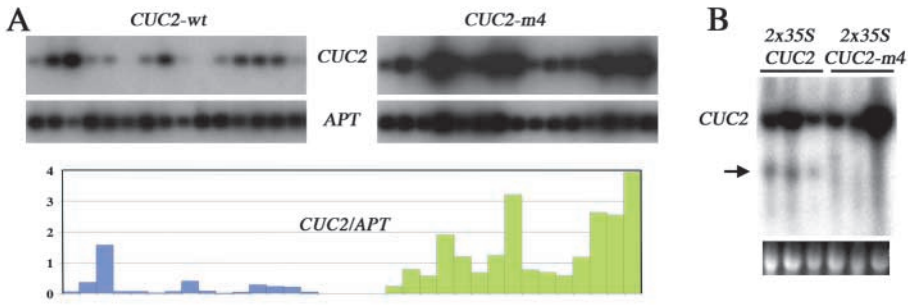

Fig. 4. *miR164*-binding is required for *CUC2* mRNA cleavage. (A) RT-PCR analysis of the *CUC2s* expression levels in *STM::ALCR-alcA::erGFP alcA::CUC2-wt* (left) and *STM::ALCR-alcA::erGFP alcA::CUC2-m4* (right) transgenic lines. Ten-day-old seedlings were sampled after overnight ethanol induction of the *CUC2-wt* or *CUC2-m4* gene. The primers used amplified RT products of the endogenous *CUC2* gene and the *CUC2-wt* or *CUC2-m4* transgenes. The same lines as in Fig. 3E were analysed and are plotted in the same order. (B) A cleavage product of *CUC2* is detected in lines overexpressing *CUC2* (*2x35S::CUC2*, arrow) and is absent in lines overexpressing the *miR164*-resistant *CUC2-m4* (*2x35S::CUC2-m4*).

site (*CUC2-c1* and *CUC2-c4*) had no effect on leaf development (Fig. 3E).

We further investigated the developmental effects of the disrupted *miR164*-binding site by analysing *STM::ALCR-alcA::erGFP* control lines and *STM::ALCR-alcA::erGFP alcA::CUC2-m4* lines that were ethanol-induced for 6 days just after bolting. No modifications of the mature flowers were observable during the first 3 weeks after induction. The mature flowers formed during the beginning of the fourth week were modified as petal number could be reduced while sepal spacing was increased (Fig. 3F).

In conclusion, these results showed that *miR164* regulation of *CUC2* was essential for plant development. In particular, disruption of the *miR164*-binding site in *CUC2* and *miR164* overexpression had opposite effects on sepal boundary formation.

The *miR164*-binding site is required for *in planta* regulation of *CUC2* messengers

We compared *CUC2* mRNA accumulation in *STM::ALCR-alcA::erGFP alcA::CUC2-wt* and *STM::ALCR-alcA::erGFP alcA::CUC2-m4* lines. Non-induced 9 day-old seedlings were ethanol-induced overnight, before tissue sampling. This short induction allowed us to compare the direct effects on *CUC2* mRNA levels by reducing secondary effects resulting from the modification of meristem organisation and thus of the size of the *STM*-expressing domain. Variable expression levels were observed between lines, but the average expression in *CUC2-m4* lines was about six times higher than in *CUC2-wt* lines (Fig. 4A). This showed that disruption of the *miR164*-binding site in *CUC2* resulted in higher mRNA accumulation levels. Accumulation of cleavage products was reported for miRNA-mediated regulation of target mRNA levels (Kasschau et al., 2003; Llave et al., 2002; Palatnik et al., 2003). We could detect a cleavage product in the *2x35S::CUC2* but not in the *2x35S::CUC2-m4* lines (Fig. 4B). No such cleavage product of *CUC2* could be detected in wild-type, *miR164* overexpressers or in the *STM::ALCR-alcA::erGFP alcA::CUC2* lines, possibly owing to low representations of the *CUC2* transcripts in these lines resulting from their localised expression (result not shown).

Expression of *miR164*-resistant *CUC2-m4* restores sepal separation in *miR164* overexpresser lines

We wanted to analyse the contribution to the floral phenotype of *miR164* overexpressers of the downregulation of the four *miR164* targets we validated (Fig. 2). We tested whether expression of *miR164*-resistant *CUC2-m4* was sufficient to restore the separation of the fused sepals of *miR164* overexpressers. Expression of only *CUC2-m4* is sufficient to test this hypothesis, as *CUC1* and *CUC2* have redundant function. We crossed the *STM::ALCR-alcA::erGFP alcA::CUC2-m4* line with strong *2x35S::miR164* lines and analysed the mature flowers following transient ethanol induction of *CUC2-m4* in the *STM*-expressing domain. One to four flowers with fully separated sepals were formed about 3 weeks after the beginning of a 5-day induction, whereas flowers with fused sepals typical of *miR164* overexpressers were present below or above the restored flowers or in the absence of ethanol induction (Fig. 5). The restored flowers showed sepal boundary enlargement characteristic of the *STM::ALCR-alcA::erGFP alcA::CUC2-m4* lines. This showed that the flower phenotype of *miR164* overexpressers could be attributed to *CUC2* and *CUC1* inactivation.

Abolition of *miR164* regulation of *CUC2* results in the progressive enlargement of the boundary domain

In order to further characterise the origin of the sepal boundary defects of the *STM::ALCR-alcA::erGFP alcA::CUC2-m4* flowers, we took advantage of the temporal control over *CUC2-m4* expression provided by the ethanol switch. We ethanol-induced the inflorescences of a *STM::ALCR-alcA::erGFP alcA::CUC2-m4* line for 6 days, and followed boundary organisation using erGFP expression under *STM* regulatory sequences as a boundary domain marker. One day after the start of induction, erGFP expression was unchanged in the *STM::ALCR-alcA::erGFP alcA::CUC2-m4* line compared with the control *STM::ALCR-alcA::erGFP* line (data not shown). After 6 days, in the control line the GFP expressing domain between emerging sepal primordia of a stage 4 flower formed a band two or three cells wide (Fig. 6, part 1), while in the *alcA::CUC2-m4* line this band was enlarged up to six or seven cells wide (Fig. 6, part 2). At 12 days, whereas in control flowers at stage 6-7 only one or two cells expressed erGFP between the sepals (Fig. 6, part 3), a group of about 10 cells could be observed in the *alcA::CUC2-m4* line (Fig. 6, part 4), showing that the boundaries were enlarged. Therefore, we concluded that expression of *miR164*-resistant *CUC2* under the control of *STM* regulatory sequences led to progressive enlargement of the boundary domain, resulting in increased spacing between sepals. This enlargement could be amplified

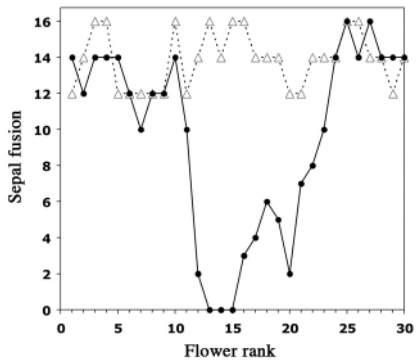

Fig. 5. Restoration of normal flower phenotype upon expression of *CUC2-m4* in *miR164* overexpressing lines. Sepal fusion was scored in the F1 progeny of a cross between a *35S::miR164* line and *STM::ALCR-alcA::erGFP alcA::CUC2-m4* line and is shown here for a representative plant that was ethanol-induced for 5 days (circles) or not induced (triangles). The degree of fusion is expressed on a scale ranging from 0 for fully separated sepals to16 for the strongest sepal fusion phenotype. Scoring on successive flowers was carried out between day 15 and 28 following induction start.

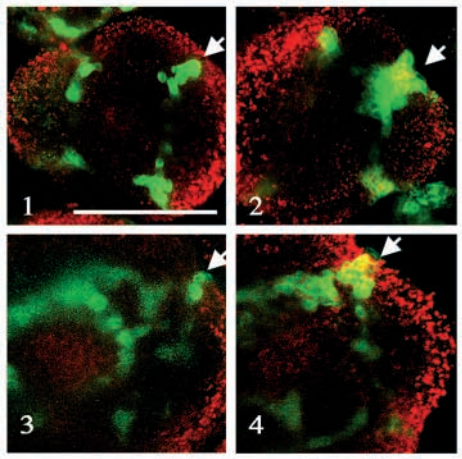

Fig. 6. Expression of *miR164*-resistant *CUC2-m4* leads to progressive boundary enlargement. Expression of erGFP under the control of *STM* regulatory sequences in developing flowers following 6 days of induction. At the end of the induction period, erGFP was expressed in a strip two or three cells wide between the sepals of a stage 4 *STM::ALCR-alcA::erGFP* flower (arrow, 1). This domain was enlarged to six to seven cells wide in a *STM::ALCR-alcA::erGFP alcA::CUC2-m4* flower at similar stage (arrow, 2). Six days later, one or two cells between the sepals expressed erGFP in a stage 6-7 *STM::ALCR-alcA::erGFP* flower (arrow, 3). In *STM::ALCR-alcA::erGFP alcA::CUC2-m4* flowers, this domain was enlarged to about 10 cells (arrow, 4). In order to realise the observations of (3) and (4), the plants had been induced again overnight to activate erGFP expression. Scale bar: 100 μm.

by the experimental approach we used: *STM* expression being activated by CUC2 (Aida et al., 1999; Daimon et al., 2003; Hibara et al., 2003; Takada et al., 2001; Vroemen et al., 2003), expression of *CUC2-m4* under the control of *STM* regulatory sequences could lead to a positive regulatory feedback loop between CUC2-m4 and the *STM* promoter. Therefore, in order to provide additional evidence that the boundary defects observed in the *miR164*-resistant *CUC2* lines were due to the disruption of the miRNA regulation of *CUC2*, we analysed the boundaries in the *dcl1*, *hen1* and *hyl1* mutants that have a general reduction of the miRNAs (Boutet et al., 2003; Chen, 2004; Han et al., 2004; Kasschau et al., 2003; Park et al., 2002; Vazquez et al., 2004). In particular, the level of *miR164* is reduced in these mutants and, accordingly, the *CUC1* and *CUC2* mRNAs levels are increased (Kasschau et al., 2003; Vazquez et al., 2004). *dcl1*, *hen1* and *hyl1* mutants show a wide range of developmental defects (Chen et al., 2002; Jacobsen et al., 1999; Lu and Fedoroff, 2000). We confirmed earlier observations that boundaries around sepals are enlarged in *dcl1* mutants (Jacobsen et al., 1999; Kasschau et al., 2003) and observed that similar defects occurred in *hyl1* and *hen1* flowers (Fig. 7). We further characterised the sepal boundaries by introducing the *STM::ALCR alcA::erGFP* in *dcl1-9* and *hyl1-1* mutants. The GFP-expressing domain around the sepals in the mutants was enlarged compared to wild type (Fig. 7). Therefore mutants having reduced *miR164* levels exhibited a similar boundary enlargement phenotype as lines expressing the *miR164*-resistant form of *CUC2* under *STM* regulatory sequences. Interestingly, the initial patterning into boundary and primordium domains is unaffected in both the miRNA mutants and the *CUC2-m4* lines, suggesting that this step is largely independent of *miR164*.

The boundary domain is a dynamic structure

We wanted to know if the enlargement of the boundary domain could be driven by the proliferation of the cells forming the boundary. Therefore, we characterised the proliferation patterns within the boundaries of the sepal primordia and compared it to the entire meristem at similar stages. We introduced a *HistoneH4::GFP* translational fusion under the control of the *alcA* promoter (*alcA::H4GFP*) into a *STM::ALCR-alcA::GUS* line (Fig. 8A, part 1). For comparison, we used a *LFY::ALCR-alcA::GUS alcA::H4GFP* line that allows expression in the entire floral meristem (Fig. 8A, part 2) (Deveaux et al., 2003). Expression of the H4GFP fusion allowed us to mark the nucleus, to recognize the mitotic figures and consequently to calculate the mitotic index (MI), with an efficiency comparable with DNA staining after plant fixation (see Fig. S2 at http://dev.biologists.org/cgi/content/full/131/17/4311/DC1).

The MI in the sepal boundary was comparable with the MI in the entire meristem and this before, during or shortly after initiation of the sepal primordia showing that proliferation is not globally repressed in the sepal boundary (Fig. 8A, part 3). The sepal boundary was further divided into two domains: a S-S domain corresponding to the boundary between two sepals and a S-M domain corresponding to the boundary between sepal and meristem (Fig. 8B, part 1). The MI in the S-S domain was 63% higher than in the S-M (Fig. 8B, part 2), showing that proliferation is differentially regulated between the different zones of the boundary, with higher proliferation rates between the sepals than between the sepals and the meristem. Interestingly, the S-S domain that shows the highest proliferation rate is also the most affected by the expression of *miR164*-resistant *CUC2-m4*. Finally, we wanted to test if divisions in the boundaries could potentially lead to their enlargement. Cells with a division axis parallel to the boundary axis, i.e. that give rise to daughter cells located in the boundary,

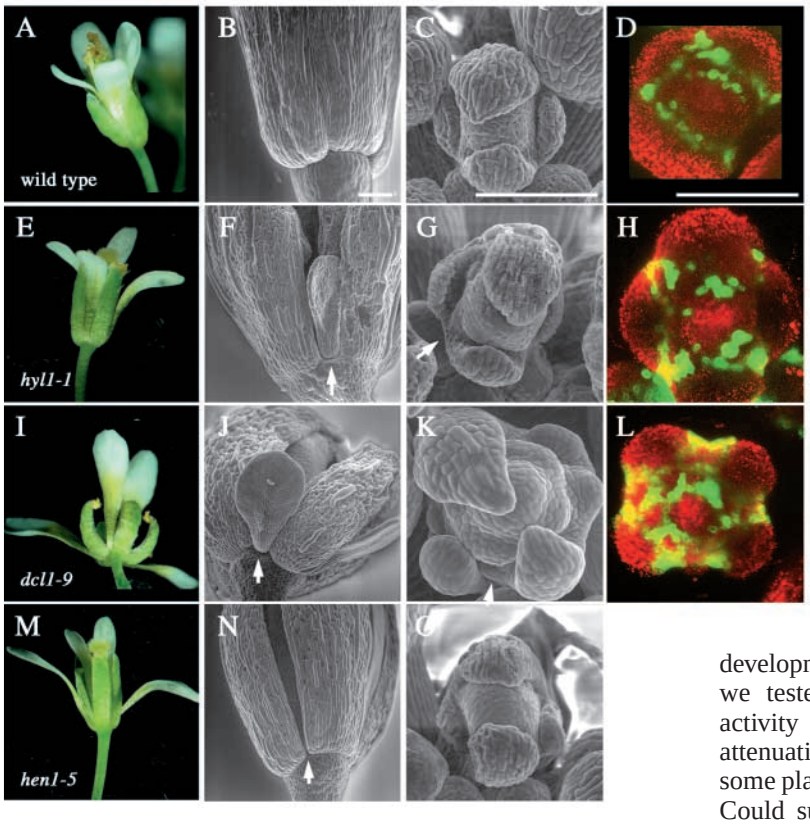

Fig. 7. The *dcl1*, *hen1* and *hyl1* mutants show boundary enlargement. Spacing of the sepals is increased in the mutants (arrows in F,J,N) compared with wild type (B). This defect is already visible at stage 5 of *hyl1-1* and *dcl1-9* mutants (arrows in G,K). The expression domain of a boundary marker (*STM::ALCR-alcA::erGFP*) is enlarged in stage 6-7 flowers of *hyl1-1* (H) and *dcl1-9* (I) mutants compared with wild type (D). Scale bars: 100 μm.

are not expected to enlarge significantly the boundary domain, whereas divisions that are orientated perpendicular to the boundary axis could induce boundary enlargement. Therefore we measured the orientation of the divisions relative to the boundary (Fig. 8C, parts 1,3). We limited these analyses to the cells of the outer cell layer as their division are only periclinal and therefore the daughter cells remain in the same layer. We did not observe any preferential division orientation of the boundary cells from the outer layer (Fig. 8C, part 3), suggesting that boundary width is not limited by a mechanism of cell division orientation control. This shows that proliferation provides a mechanism by which the sepal/sepal boundary can enlarge and that is controlled by *miR164*-dependant regulation of *CUC1* and *CUC2*.

Discussion

We show that *miR164* coded by two loci, *MIR164A* and *MIR164B*, post-transcriptionally degrades *CUC1* and *CUC2* mRNAs. Modification of the regulatory relationship between *miR164* and the targets, either by increasing or reducing *miR164* level or by making the *CUC2* target resistant to it, leads to abnormal boundary size regulation. Our cellular analysis of the sepal boundaries shows that they are not maintained via a control of the proliferation patterns but at least in part by *miR164*-mediated degradation of *CUC1* and *CUC2* mRNAs, thus demonstrating the involvement of miRNAs in the control of developmental patterns.

By overproducing *miR164*, we showed that this miRNA reduced the mRNA level of four out of the five predicted targets (Rhoades et al., 2002). This downregulation resulted from miRNA-guided cleavage of the mRNA, as we could detect degradation products of the *CUC2* mRNA that were dependent on the presence of a *miR164* complementary site in *CUC2*, confirming previous identification of *CUC1* and *CUC2* mRNA cleavage products (Kasschau et al., 2003). We did not observe a reduction of *NAC1* mRNA level in inflorescences of *miR164* overexpressers. Several hypotheses could account for this. *NAC1* may not be a real target of *miR164*. *miR164*-mediated degradation of *NAC1* mRNA could also be developmentally regulated and not occur under the conditions we tested. Alternatively, *miR164* may not regulate *NAC1* activity via mRNA cleavage but through translational attenuation as generally observed for animal miRNA and for some plant miRNAs (Aukerman and Sakai, 2003; Chen, 2004). Could such a dual mechanism for *miR164* be the result of differences in the target sequences? Two or three mismatches are observed between *miR164* and the five predicted targets (Rhoades et al., 2002). However, if pairings between G and U are allowed, two mismatches subsist for *NAC1-miR164* complexes, whereas only one exists for the four targets for which cleavage is observed. It would therefore suggest that the mode of action of *miR164* depends on the extent of its homology with the target, as observed for small RNAs in animals (Doench et al., 2003; Zeng et al., 2003). It must be noted that, although G-U base pairing is possible, our mutational analysis of the *miR164*-binding site of *CUC2* (*CUC2-m1*) showed that G-U pairing is not functionally equivalent to G-C pairing.

We show that during early phases of sepal boundary development, cell proliferation is not repressed and that there is no strict restriction of cell division orientations. Therefore, transverse cell division that can potentially lead to boundary enlargement can occur unless the boundary identity is rapidly switched off. We provide evidence that *miR164*-dependent degradation of *CUC1* and *CUC2* transcripts constrains the expansion of the boundary domain resulting from boundary cell proliferation. First, boundary enlargement was observed when a *miR164*-resistant *CUC2* form was expressed in the boundary domain using *STM* regulatory sequences. Second, similar boundary defects were observed in mutants with reduced *miR164* levels. What is the relation of *miR164*-dependant boundary size regulation with cell proliferation? *MiR164* may switch off the *CUC1,2* function after division in one of the daughter cells and thus induce different cell identities. Alternatively, the link with cell division could be looser. *miR164* may switch off the *CUC1,2* function in the outermost boundary cell in response to boundary enlargement, resulting either from the proliferation of this cell or from

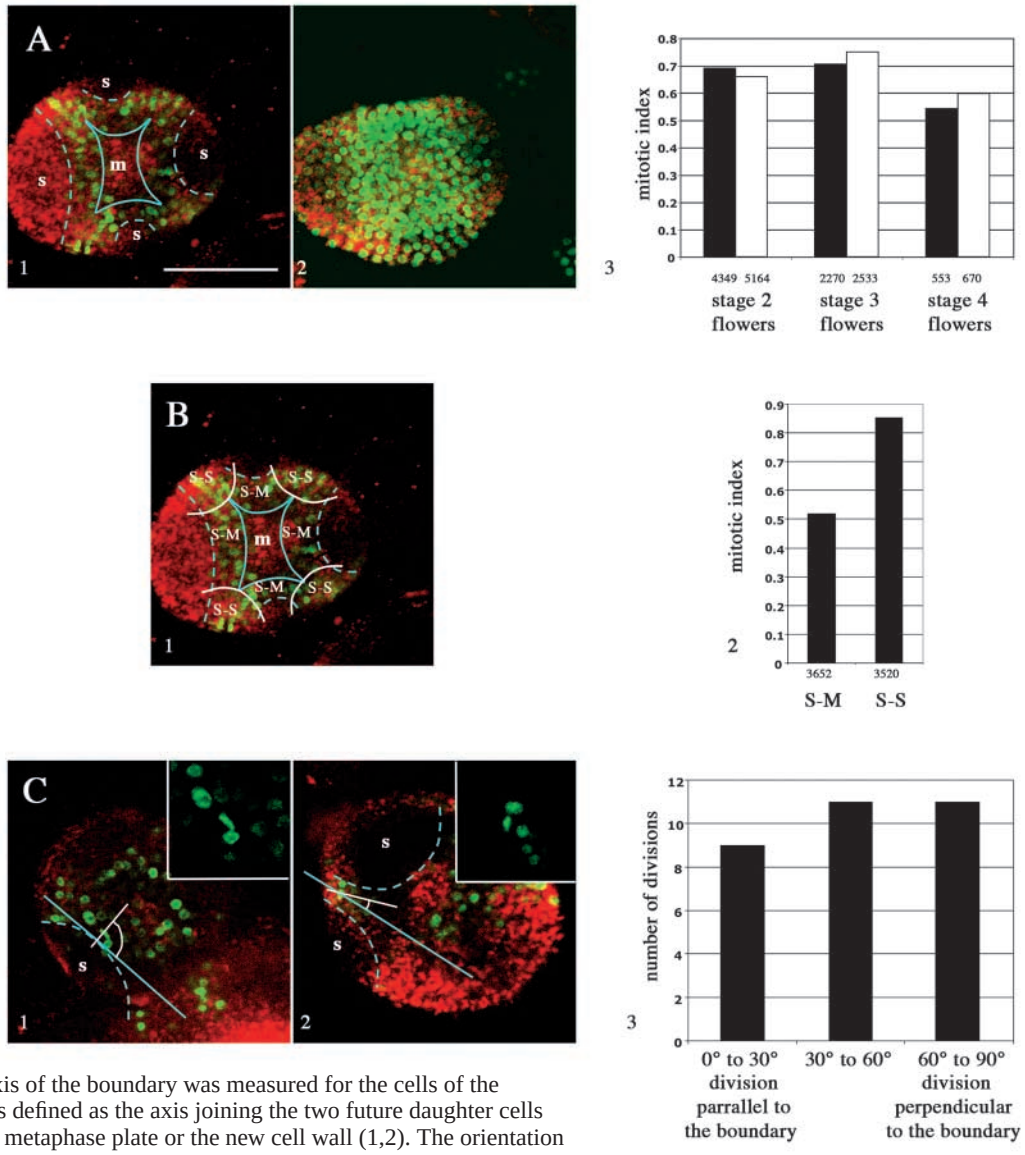

Fig. 8. Proliferation in the sepal boundaries. (A) An *alcA::HistoneH4GFP* (*alcA::H4GFP*) construct was introduced into a *STM::ALCR alcA::GUS* line (1) or into a *LFY::ALCR alcA::GUS* line (2) in order to obtain inducible H4GFP expression in the boundary domain or in the entire meristem, respectively. As *STM*-driven expression of the *H4GFP* extended towards the centre of stage 2 meristems, we considered as part of the boundary only the two marked cells files closest to the primordium (between broken and unbroken blue lines in 1). (3) The mitotic index (MI) in the sepal boundary domain (black bars) and in the entire meristem (white bars) was calculated for floral meristems before (stage 2) during (stage 3) or just after (stage 4) sepal primordia initiation. The four outermost cell layers were analysed and the number of cell counted for each class is indicated below the bars. (B) The sepal boundaries of stage 2-4 flowers were subdivided into boundaries between two sepals (S-S) or between sepals and the meristem (S-M) (1). (2) The MI of these two domains was calculated. The four outermost cell layers were analysed and the number of cells counted for each class is indicated below the bars. (C) The orientation of the division axis of dividing cells relative to the axis of the boundary was measured for the cells of the outermost layer. The division axis was defined as the axis joining the two future daughter cells and is perpendicular to the axis of the metaphase plate or the new cell wall (1,2). The orientation of a S-M dividing cell was calculated relative to the boundary axis (1). The boundary axis was defined as a line tangent to the outer limit of the boundary domain (recognisable as the limit between GFP-expressing and non-expressing cells). The orientation of a S-S dividing cell was calculated relative to the two adjacent boundaries (2). The insets (1,2) show magnifications of the dividing cell. (3) The number of dividing cells was plotted against the orientation of the division axis. Orientations with a high angle value (see 1) are perpendicular to the boundary whereas low values (see 2) are parallel. m, meristem centre; s, sepal primordium.

another cell. In both cases, a boundary cell would reset its identity and adopt either a meristem or a primordium identity. The latter could account for the earlier observation that, during pea leaf development, cells are recruited into the growing primordium from adjacent domains (Lyndon, 1970).

miRNAs are evolutionary conserved in both plants and animals. miR164 homologues have been reported for rice and tobacco (Mallory et al., 2002; Reinhart et al., 2002), and could be found in database for poplar and *Medicago truncatula*. In addition to *Arabidopsis*, a potential *miR164*-binding site is present in *NAC* genes of rice (Rhoades et al., 2002), petunia, *Antirrhinum majus*, soybean and bean. At least two of them, *NAM* and *CUP*, have a similar role to the Arabidopsis CUC genes in petunia and *Antirrhinum*, respectively (Souer et al., 1996; Weir et al., 2004). Therefore, the mechanism of boundary stabilisation we described for *Arabidopsis* is likely to be evolutionary conserved. A similar conservation of the miRNA-target function has been described for the control of leaf polarity between *Arabidopsis* and maize (Floyd and Bowman, 2004; Juarez et al., 2004; Kidner and Martienssen, 2004).

We have shown that *CUC1* and *CUC2* mRNAs are targeted for degradation by *miR164*, whereas *CUC3* is not directly regulated by the miRNA. Why is *CUC3* not a target of *miR164*? A higher level of redundancy seems to exist within the CUC genes in *Arabidopsis* than in other species (Souer et al., 1996; Weir et al., 2004). Nevertheless, although the *CUC1*, *CUC2* and *CUC3* genes have all a role in boundary specification, their contribution is not identical. First, genetic analyses suggest that the contribution of *CUC3* to cotyledonary

boundaries is more important than those of *CUC1* and *CUC2* (Vroemen et al., 2003). Second, expression patterns of the *CUC1*, *CUC2* and *CUC3* genes differ slightly during embryogenesis (Vroemen et al., 2003). Finally, the *CUC2* expression domain is reduced in the embryo of the *pin-formed1* mutant, whereas the *CUC1* domain is expanded (Aida et al., 2002), suggesting that these two genes differ in their response to the signalling molecule auxin involved in primordia patterning (Reinhardt et al., 2003). It appears therefore that the precise regulation of *CUC1*, *CUC2* and *CUC3* involves different mechanisms. In this context, miRNA-regulation is apparently an additional level of control. Besides, it is possible that another, not yet identified, miRNA could regulate *CUC3*.

Note added in proof

While this paper was under review, Mallory et al. (Mallory et al., 2004) described partially overlapping results.

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