A role for chromatin remodeling in regulation of CUC gene expression in the Arabidopsis cotyledon boundary

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The CUP-SHAPED COTYLEDON (CUC) genes CUC1, CUC2 and CUC3 act redundantly to control cotyledon separation in Arabidopsis. In order to identify novel regulators of this process, we have performed a phenotypical enhancer screen using a null allele of cuc2, cuc2-1. We identified three nonsense alleles of AtBRM, an Arabidopsis SWI/SNF chromatin remodeling ATPase, that result in strong cotyledon fusion in cuc2-1. Atbrm also enhances cotyledon fusion in loss-of-function cuc1 and cuc3 mutants, suggesting a general requirement for this ATPase in cotyledon separation. By contrast, a null allele of SPLA YED (SYD), the closest homolog of AtBRM in Arabidopsis, enhances only the loss-of-function cuc1 mutant. By investigating the activities of the CUC promoters in the cotyledon boundary during embryogenesis in sensitized backgrounds, we demonstrate that AtBRM upregulates the transcription of all three CUC genes, whereas SYD upregulates the expression of CUC2. Our results uncover a specific role for both chromatin remodeling ATPases in the formation and/or maintenance of boundary cells during embryogenesis.

KEY WORDS: Arabidopsis, Embryo, Boundary formation, CUP-SHAPED COTYLEDON, Chromatin remodeling ATPase

INTRODUCTION

In plants, establishment of the boundary region is crucial for the development of separate rather than fused organs from primordia, affecting overall plant form and architecture (Aida and Tasaka, 2006). In dicotyledonous plants, globular embryos with radial symmetry develop into heart-shaped embryos with bilateral symmetry. During this transition, the cotyledon boundary and the shoot apical meristem (SAM) are formed between the two outgrowing cotyledon primordia (Aida and Tasaka, 2006). Mutant plant seedlings that exhibit complete fusion between the cotyledons generally fail to establish a SAM during embryogenesis (Aida et al., 1997; Souer et al., 1996; Vroemen et al., 2003; Weir et al., 2004). Thus, factors controlling boundary formation between the cotyledons are also required for establishment of the SAM. As the SAM ultimately gives rise to all above-ground plant structures, proper establishment of the cotyledon boundary is thus crucial for postembryonic development (Aida and Tasaka, 2006).

Genetic screens aimed at identifying mutations that lead to fusions between adjacent organs revealed that a class of NAC domain transcription factors (Olsen et al., 2005) are central regulators of organ boundary formation in plants. The developmentally characterized NAC domain genes relevant to boundary formation include NO APICAL MERISTEM (NAM) in petunia (Souer et al., 1996), CUPULIFORMIS (CUP) in snapdragon (Weir et al., 2004) and the CUP-SHAPED COTYLEDON (CUC) genes CUC1, CUC2 and CUC3 in Arabidopsis (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). The three CUC genes in Arabidopsis are closely related and play functionally redundant roles in organ boundary formation. They are expressed in an overlapping pattern in the cotyledon boundary, a stripe of cells between the incipient cotyledon primordia in early heart stage embryos (Aida et al., 1999; Takada et al., 2001; Vroemen et al., 2003). CUC genes are also expressed after embryogenesis in leaf boundaries (Vroemen et al., 2003), as well as in the boundaries between floral organs (Baker et al., 2005; Takada et al., 2001; Vroemen et al., 2003). Single cuc mutants exhibit either no phenotype or very subtle phenotypes, whereas loss-of-function mutations in any combination of two different CUC genes lead to defects in boundary formation and hence fusion of the cotyledon margins, resulting in striking cup-shaped seedlings (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003).

Little is known about the positive regulators of CUC gene expression that are essential for CUC-dependent formation and/or the maintenance of boundary cells. The SHOOT MERISTEMLESS (STM) transcription factor is one candidate activator of CUC1 function (Aida et al., 1999; Aida et al., 2002). Expression of STM, in turn, is dependent on presence of the CUC gene products (Aida et al., 1999). In an attempt to identify additional positive regulators of CUC expression and/or activity, we performed an enhancer screen of the cuc2-1 null allele. Our genetic screen was designed to identify novel factors that function redundantly with CUC2. We identified three nonsense mutations in AtBRM as genetic enhancers of cotyledon fusion. AtBRM is one of four SWI/SNF chromatin remodeling ATPases in Arabidopsis (Farrona et al., 2004; Su et al., 2006; Wagner and Meyerowitz, 2002) (www.chromdb.org). In addition, we show that AtBRM is required in cuc2-1 for transcription of all three CUC genes in the cotyledon boundary. By contrast, mutants of SYD, the closest relative of AtBRM in Arabidopsis, enhance cotyledon fusion of cuc1-1 mutants where SYD is required for CUC2 transcription.

SWI/SNF chromatin remodeling ATPases are structurally and functionally conserved from yeast to mammals, and are implicated in transcriptional regulation. The ATPases are generally recruited to specific promoters by transcriptional regulators (reviewed by de la Serna et al., 2006; Kadam and Emerson, 2002; Simone, 2006), where they remodel DNA/histone octamer interactions to regulate the accessibility of binding sites to transcriptional regulators and/or the general transcriptional machinery (see Fan et al., 2004). They are subunits of large multiprotein complexes with different tissue and cell type-specific composition (e.g. Eberharter et al., 2005; Mohrmann and Verrijzer, 2005). Although the mechanism of action

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and the complex composition of the SWI/SNF remodelers is becoming better understood, understanding their role in the organism has been hampered by the fact that mutations in most SWI/SNF ATPases are embryo lethal (Bulman et al., 2000; Bulman et al., 2005; Indra et al., 2005; Reyes et al., 1998; Sawa et al., 2000; Tamkun et al., 1992). We show here that the two SWI/SNF ATPases AtBRM and SYD are specifically and differentially required for cotyledon separation in Arabidopsis via the regulation of expression of a small gene family.

MATERIALS AND METHODS

Plant lines and growth conditions

cuc2-1, stm-1, stm-2, cuc1-1 and syd-2 mutants have been described (Aida et al., 1997; Barton and Poethig, 1993; Clark et al., 1996; Takada et al., 2001; Wagner and Meyerowitz, 2002). stm-2 syd-2 was described in a previous study (Kwon et al., 2005). cuc3-101, isolated as an enhancer mutant of cuc2-1, carries a point mutation at the junction of the first exon and intron (AG:GT to AG:AT) and represents a strong allele (K.i.-H., M.A. and M.T., unpublished). Double-mutant plants made in this study were genotyped for cuc1-1, cuc3-101, syd-1, stm-2, atbrm-1, atbrm-2 and atbrm-3 using dCAPS. Homozygous cuc2-1 was genotyped by amplifying a genomic DNA region flanking the transposon insertion site (Aida et al., 1997). Segregating populations of syd-2 and atbrm-1 were individually maintained in Ler, cuc1-1, cuc3-101 and cuc3-101 homozygous backgrounds. stm-2 atbrm-1 was maintained as double heterozygotes. Seeds were sown on sterilized soil mix (Promix BX; Premier Horticulture, Quakertown, PA), stratified in the dark at 4°C for 7 days, and then grown at 22°C in long day (16 hours light) conditions at 120 μmol/m²·sec of cool white light. Plants were photographed using an Olympus SZX12 dissecting microscope equipped with a Spot Insight camera (Diagnostic).

Fusion of floral organs was determined for the first ten flowers formed on seven individual plants. We predominantly observed fused sepals and fused stamen filaments.

Real-time PCR

Total RNA was isolated from the above-ground parts of seedlings using a RNeasy Plant mini kit with on-column DNA digestion (Qiagen). First strand cDNA synthesis was performed with an oligo dT primer using a Thermoscript kit (Invitrogen). Quantitative real-time PCR was performed in a 20 μl PCR reaction using the QuantiTect SYBR Green PCR kit (Qiagen) on a DNA Engine Opticon Thermal cycler (MJ Research). Thermal cycling conditions were as follows: 16 minutes at 95°C, and then 45 cycles of 15 seconds at 94°C, 30 seconds at 56°C and 30 seconds at 72°C, followed by melting curve analysis. The data obtained was analyzed with the Opticon Monitor Analysis Software (Version 1.4). Relative amounts of all mRNA were calculated from threshold cycle values and standard curves, and normalized with the level of eukaryotic translation initiation factor 4A-1 (EIF4A). Specificity of real-time PCR products was confirmed by electrophoresis on a 2.5% agarose gel. Primers used were described previously (Kwon et al., 2005). In addition, we employed the following primers:

**ANT**, TGGAACTTTTGGAAACCCAG and CTTGGGTTCACA-AAAGTTCCA;
**FIL**, AACCATCCTTGCGTTAATG and TTAACGGGTGGTGCTTAGG; and
** UFO**, TCTGGGCCGATGCATTCTTCTT and ATCAAAACAGCAACTGCAAGC.

Transgenic plants

A 1.4 kb 5’ intergenic region of CUC1 similar to that used for complementation (Baker et al., 2005) was placed upstream of the GUS gene in pb1101 (Clontech) for CUC1::GUS expression. A 3.2 kb 5’ intergenic and a 2.5 kb 3’ intergenic region of CUC2, and a 4.3 kb 5’ intergenic and a 2 kb 3’ intergenic region of CUC3, were placed upstream and downstream of GUS gene in pb1101 for CUC2::GUS and CUC3::GUS expression, respectively. The resulting plant binary vectors were electroporated into Agrobacterium LBA4404 (Clontech), and transformed into Arabidopsis plants as previously described (Clough and Bent, 1998). The transgenes harboring CUC::GUS were individually introgressed into cuc1-1 syd-2/+ and cuc2-1 atbrm-1/+ by crossing, and single locus homozygotes for the respective transgene were used for GUS experiments.

GUS assays

During harvesting, embryos were incubated in 7% glucose solution at room temperature. Embryos were fixed in 90% acetone for 20 minutes on ice, and washed with GUS staining buffer [0.2% Triton X-100, 2 mM potassium ferrocyanide/ferrocyanide, 50 mM sodium phosphate buffer (pH 7.2)]. After vacuum infiltration for one minute in the GUS staining buffer with 2 mM X-glucuronide (Gold BioTechnology), GUS assays were performed from one to three hours at 37°C, or overnight at 30°C, depending on CUC promoter activity in the individual transgenic line, and then stored in 70% ethanol for microscopic analysis. GUS signals were photographed with darkfield illumination using an Olympus BX51 compound microscope.

RESULTS

Isolation of atbrm as an enhancer of cuc2-1 in cotyledon fusion

In order to identify factors that function redundantly with one of the CUC genes, we screened for seedlings with fused cotyledons in the M2 progeny of 3500 EMS-mutagenized CUC2 null mutants (cuc2-1; Fig. 1A) (Aida et al., 1997). Fourteen enhancers were identified that displayed a fused cotyledon phenotype at variable frequencies (K.i.-H., M.A. and M.T., unpublished). Among these, three mutants were found to fall into one complementation group (L77, T59, H48). All three showed fused cotyledon phenotypes in the cuc2-1 background with a similar penetrance. These mutants also shared additional phenotypes, such as short roots, short stature, and female and male sterility (see below). The mutation in H48 was mapped to a 106.1-kbp region that spans the BACs F4118 and T3F17 in chromosome 2. Sequence analysis of the AtBRM gene, which resides within this region, identified nonsense mutations in the L77, T59 and H48 mutants. The mutants were therefore designated atbrm-1, atbrm-2 and atbrm-3, respectively (Fig. 1G,H). To confirm that mutations in AtBRM act as phenotypic enhancers of cuc2, we obtained the SALK_030046 line (Alonso et al., 2003), which carries a T-DNA insertion immediately after codon 41 of AtBRM, and designated this allele atbrm-4 (Fig. 1G). The atbrm-4 mutant also caused frequent cotyledon fusion when introduced to the cuc2 mutant background (data not shown). We conclude that mutations in AtBRM enhance the cotyledon boundary defect of the cuc2 mutant.

Among the four atbrm mutants identified, atbrm-1 is predicted to produce a truncated protein that does not contain any of the conserved motifs found in SWI/SNF ATPases (Farrona et al., 2004; Mohrmann and Verrijzer, 2005; Tsukiyama, 2002; Wagner and Meyerowitz, 2002), and is therefore likely to be a null allele (Fig. 1H). cuc2-1 atbrm-1 double mutants form cup-shaped seedlings at a high frequency (Fig. 1D, red arrowheads; see also Fig. 3), while less frequently fusions of one cotyledon margin were observed (Fig. 1E, red arrowheads; Fig. 3). atbrm-3 is predicted to lack three of the five conserved domains in SWI/SNF ATPases, including part of the catalytic domain (Fig. 1H). cuc2-1 atbrm-3 mutants showed similar cotyledon separation defects to cuc2-1 atbrm-1 mutants (compare Fig. 1D with 1F), confirming that the conserved domains affected in atbrm-3 are important for AtBRM activity.

Other phenotypes of the three atbrm alleles were similar to (albeit more severe than) those previously described for knockdown RNAi alleles (Fig. 2) (Farrona et al., 2004). atbrm-1 null mutants are small (Fig. 1B, Fig. 2A), slow growing, and have downwards curled leaves (Fig. 2A). atbrm-1 flowers fail to open

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investigate the functional overlap between the two ATPases in the regulation of Arabidopsis development. SYD, the closest homolog of AtBRM, may also be involved in the formation of the cotyledon boundary based on the enhanced cotyledon fusion observed in smn-2 syd-2 double mutants (Kwon et al., 2005) (and see below). SYD and AtBRM are closely related (Su et al., 2006) (http://www.chromdb.org/) with 50% amino acid identity in the ATPase domain (Farrona et al., 2004). AtBRM and SYD share conserved domains I and II (N-terminal region), and the AT-hooks (downstream of the catalytic domain) (Farrona et al., 2004; Wagner and Meyerowitz, 2002). AtBRM alone has a fifth domain conserved in metazoan SWI/SNF ATPases, the bromodomain (Farrona et al., 2004). By contrast, SYD has a divergent C-terminal domain (Wagner and Meyerowitz, 2002), which is not required for biological activity (Su et al., 2006).

In order to determine whether the syd-2 and atbrm-1 null mutants cause cotyledon fusion in all three cuC mutants, we constructed the relevant double mutants with the strong/null alleles cuC1-1, cuC2-1 and cuC3-101 (Aida et al., 1997; Takada et al., 2001) (K.i.-H., M.A. and M.T., unpublished). We observed a marked enhancement in the frequency and extent of cotyledon fusion in all three cuC atbrm double mutant combinations, with the greatest degree of fusion observed in cuC2-1 atbrm-1 (Fig. 3F). By contrast, cuC2-1 strongly enhanced the cotyledon fusion of cuC1-1, had only a very modest effect on cuC3-101, and had no effect on cuC2-1 (compare Fig. 3B,C with 3A; Fig. 3F). In agreement with this data (although our genetic screen was likely not saturating), we did not identify syd mutations as enhancers of cuC2-1 (K.i.-H., M.A. and M.T., unpublished).

The strong cotyledon fusion in cuC1-1 syd-2 relative to cuC2-1 syd-2 and cuC3-101 syd-2 (Fig. 3F) suggests that SYD does not act via cuC1. As cuC1-1 is a null mutation or a very strong loss-of-function mutation (Takada et al., 2001), the enhanced phenotype of cuC1-1 syd-2 is likely to result from the effect of syd-2 on genes that function in parallel to cuC1, such as cuC2 or cuC3. Because cuC2-1 syd-2 plants were indistinguishable from the parental lines with respect to cotyledon fusion, SYD is predicted to primarily act via cuC2. By contrast, the phenotypical analysis of double mutants between cuC and atbrm-1 suggests that AtBRM acts on at least two of the CUC genes: all double mutant combinations resulted in a strong enhancement of cotyledon fusion (Fig. 3F).

In addition to boundary establishment between the cotyledons, the CUC gene products also play a role in shoot apical meristem (SAM) formation during embryo development (Aida et al., 1999; Hibara et al., 2003; Takada et al., 2001). Most cuC1-1 syd-2 plants showing cotyledon fusion are heart-shaped in appearance but do not form shoots (Fig. 1D). Shoots do not form even in severe cuC1-1 cuC3-101 syd-2 mutants (Fig. 1E). In these heart-shaped seedlings, shoots are readily formed inside the fused petiole (Fig. 3D) and are visible after penetrating the petiole (Fig. 3C), indicating there is no substantial defect in SAM formation. Shoots can also be formed in severe cup-shaped seedlings of cuC1-1 syd-2, cuC1-1 atbrm-1 and cuC3-101 atbrm-1 (Fig. 3C-E; data not shown). By contrast, cup-shaped seedlings of cuC2-1 atbrm-1 do not form shoots (Fig. 1D), suggesting a loss of SAM activity. The SAM loss in cuC2-1 atbrm-1 correlates with the very strong cotyledon fusion observed in these double mutants. In addition, because cuC2 is thought to play a more central role in the formation of SAM than cuC1 and cuC3 (Vroemen et al., 2003), the complete loss of cuC2 activity in cuC2-1 atbrm-1 may contribute to the loss of SAM formation.

**Distinct genetic interactions of atbrm-1 and syd-2 with individual cuC mutants**

*Arabidopsis* has four SWI/SNF ATPases (see http://www.chromdb.org/): AtBRM, SYD, CHR12 and CHR23. The identification of null mutants in two of these, AtBRM (Figs 1, 2) and SYD (Wagner and Meyerowitz, 2002) allowed us to
Both atbrm-1 and syd-2 mutants enhance cotyledon fusion in the shoot meristemless-2 background

Individual cuc mutants enhance cotyledon fusion in shoot meristemless (sm) mutants (Aida et al., 1999). We have previously shown that syd-2 enhances the cotyledon fusion of sm-2, albeit less efficiently than do cuc mutations (Aida et al., 1999; Kwon et al., 2005) (compare Fig. 4A with 4D). sm-2 atbrm-1 (Fig. 4B) exhibits a much stronger cotyledon fusion defect than sm-2 syd-2, consistent with the conclusion that AtBRM has a broader role in cotyledon boundary formation than SYD.

Because of the compromised SAM activity in sm mutants, very few leaves are formed in the weak sm-2 allele, and no leaves are formed in the strong sm-1 allele, or in sm-2 syd-2 or sm-2 atbrm-1 double mutant seedlings (Barton and Poethig, 1993; Clark et al., 1996; Kwon et al., 2005). CUC mRNA expression in these double mutants is thus largely due to expression in the cotyledon boundary rather than to expression in the leaf boundary. To determine whether SYD and AtBRM regulate CUC gene expression in the cotyledon boundary, we utilized the sm-2 mutant to examine CUC1, CUC2 and CUC3 levels in sm-2 syd-2 and sm-2 atbrm-1 cotyledon boundaries by real-time RT-PCR (Fig. 4E). Compared with sm-2 and sm-1, the expression level of all three CUC genes is reduced in sm-2 atbrm-1. By contrast, expression of CUC2 and CUC3, but not of CUC1 is reduced in sm-2 syd-2, in agreement with our previous data (Kwon et al., 2005) (Fig. 4E). The slight increase in CUC1 message in sm-2 syd-2 compared with in sm-2 may be due to CUC1 expression in the fused cotyledon margin (see Fig. 5M). Although the CUC1 message reduction in sm-2 atbrm-1 is significant when compared with the parental sm-2 and the strong sm-1 allele, it is not as strong as the profound reduction of CUC2 and CUC3 in this line (Fig. 4E). Consistent with the genetic interactions, these expression studies suggest that AtBRM may regulate the expression of all three CUC genes, whereas SYD appears to promote the expression of CUC2 and CUC3, but not CUC1.

To examine whether the effect of SYD and AtBRM on CUC gene expression is specific, we next analyzed the expression of genes implicated in the patterning of the embryonic meristem and cotyledons (Bowman and Eshed, 2000; Watanabe and Okada, 2003). We were unable to detect a significant alteration in the expression of FILAMENTOUS FLOWER (FIL), AINTEGUMENTA (ANT) or UNUSUAL FLORAL ORGANS (UFO) in sm-2 syd-2 or sm-2 atbrm-2 when compared with sm-2 (Fig. 4F), suggesting that radial patterning of the shoot apex is not affected in the double mutants.

Fig. 2. Phenotypes of atbrm and syd. (A) atbrm-1 mutant (top) has a smaller rosette and curled leaves compared with the co-segregating wild-type plant (bottom). (B,C) Whereas the flowers in the wild-type inflorescence are completely open (C), flowers remain closed in the atbrm-1 inflorescence (B). (D,E) Fused stamen filaments (red arrowheads) can be found in the flowers of atbrm-1 (D) and syd-2 (E). (F) Root growth is retarded in atbrm-3 seedlings (left) relative to the wild type (right). Scale bars in B,C: 2.5 mm.

Fig. 3. Phenotypic analyses of cuc and syd-2 or atbrm-1 double mutants. (A-C,E) Pictures taken at the indicated days after germination. None of the double mutants exhibit loss of shoot apical meristem function with the exception of cuc2-1 atbrm-1 (not shown); shoots form from the fused region of the cotyledons (yellow arrowheads in C-E). Genotypes are indicated in each panel. (D) Picture of a cleared seedling taken using DIC optics. Scale bars: 2 mm in A-C,E, 100 µm in D. (F) Percentage of plants showing cotyledon fusion in double mutant combination with cuc1-1, cuc2-1 or cuc3-101. Cup indicates cup-shaped seedling with substantial fusion on both sides of each cotyledon blade (as in B and E). CPF includes seedlings with complete fusion of one side of the cotyledon blade (see C), fusion of the cotyledon petiole, as well as cup-shaped seedlings. atbrm-1 mutants enhance all three cuc mutants, whereas syd-2 mutants only significantly enhance cuc1-1. The number of seedlings tested for each genotype is shown in parentheses.
We also examined CUC expression in cuc2-1 atbrm-1 and cuc1-1 syd-2 seedlings, which display very strong cotyledon fusion (Fig. 3F). We observed a similar reduction in CUC gene expression to that described above for stm-2 atbrm-1 and stm-2 syd-2 (see Fig. S1 in the supplementary material), further supporting a role of AtBRM and SYD in the regulation of this small gene family. We did not detect a significant reduction of CUC gene expression in atbrm-1 or syd-2 compared with wild-type seedlings (Fig. S1 in the supplementary material). This may be due to functional redundancy of SYD and AtBRM with the two additional SWI/SNF ATPases in Arabidopsis (www.chromdb.org), or due to SYD and AtBRM independent expression of the CUC genes in other boundaries besides the cotyledon boundary in the single mutant seedlings. However, organ fusion defects can be observed in syd-2 and atbrm-1 single mutants, supporting the conclusion that both ATPases are required for organ boundary formation in Arabidopsis. For example, a low but significant incidence of cotyledon fusion is noticeable in atbrm-1 (Fig. 3F). In addition, syd-2 flowers often have fused sepals (Wagner and Meyerowitz, 2002), and both atbrm-1 and syd-2 mutants exhibit stamen filament fusion (Fig. 2D,E). Forty percent of syd-2 flowers and 53% of atbrm-1 flowers display organ fusions. These types of defect are commonly observed when CUC expression is reduced in the flower (Aida et al., 1997; Baker et al., 2005; Mallory et al., 2004). Consistent with this notion, we observed a reduction in CUC2 expression in atbrm-1 and syd-2 single mutant inflorescences when compared with the wild type (Fig. 4G).

**AtBRM and SYD are differentially required for CUC gene expression during embryogenesis**

To examine the role of AtBRM and SYD in the cotyledon boundary during embryonic development, we generated reporter constructs that faithfully reproduce the expression of the three CUC genes (Fig. 5B,D-FJ-L) (Aida et al., 1999; Aida et al., 2002; Vroemen et al., 2003). We investigated the activities of CUC promoters driving β-glucuronidase (GUS) in cuc2-1 atbrm-1 and cuc1-1 syd-2 double mutant embryos (compare left with right embryo in Fig. 5C, as well as Fig. 5G with 5D, 5H with 5E, and 5I with 5F for atbrm-1 mutants; and 5M with 5J, 5N with 5K, and 5O with 5L for syd-2 mutants). Partially fused (Fig. 5C,G,H) or fully fused (Fig. 5I) cuc2-1 atbrm-1 embryos showed reduced expression of CUC1::GUS, CUC2::GUS and CUC3::GUS reporters in the presumptive boundary cells, when compared with the strong expression of these reporters in non-fused regions (Fig. 5C,G,H; data not shown). Thus, AtBRM controls the transcription of all three CUC genes required for proper cotyledon boundary formation.

Partially fused (Fig. 5M,O) or fully fused (Fig. 5N) cuc1-1 syd-2 embryos showed no CUC2::GUS expression in the presumptive boundary cells between the cotyledons (Fig. 5N), but showed expression of CUC1::GUS and CUC3::GUS (Fig. 5M,O, red arrowheads; data not shown). This finding supports the conclusion that SYD regulates the expression of CUC2 but not CUC1, and is consistent with the molecular defects observed in stm-2 syd-2 (Fig. 4). Because SYD is not required for CUC3 expression during embryo development, according to the reporter studies, yet stm-2 syd-2 seedlings showed reduced CUC3 expression, it is possible that the latter is an indirect effect observed in later stages of development. We conclude that SYD promotes the expression of CUC2 during cotyledon boundary formation. Our data further indicate that the regulatory sequences upon which AtBRM and SYD act are present in the intergenic sequences used for the reporter studies.

Cotyledon fusion does not always result in a loss of CUC expression; CUC1::GUS and CUC3::GUS activities can be observed in the fused region of cuc1-1 syd-2 embryos (Fig. 5M,O). Therefore, the strongly decreased CUC promoter activities in the fused region of cuc2-1 atbrm-1 (as well as the reduction of CUC2 promoter activity in cuc1-1 syd-2) are likely to be the cause rather
than the consequence of the cotyledon fusion events (Fig. 5C,G,H,I,N). It is difficult to examine the direct in vivo interaction of SYD and/or AtBRM with regulatory regions of the CUC promoters because of the small number of cells contributing to the cotyledon boundary in embryos (Fig. 5). It is therefore not clear whether SYD and AtBRM act directly on the CUC genes or further upstream.

DISCUSSION
Roles of AtBRM and SYD in the cotyledon boundary
We have identified AtBRM as a new positive regulator of CUC expression. Mutations in the chromatin remodeling ATPase AtBRM enhance cotyledon fusion in combination with mutants of any of the three CUC genes. Moreover, AtBRM is required for the proper expression of CUC1, CUC2 and CUC3. By contrast, the closely related chromatin remodeling ATPase SYD is required for expression of CUC2, and the syd-2 mutant enhances cotyledon fusion in the cuc1 mutant. Our genetic and molecular data indicate that chromatin-mediated control of gene expression is important for proper cotyledon separation and that this role is fulfilled by two closely related chromatin remodeling ATPases. These ATPases have overlapping functions: CUC2 is regulated by both SYD and AtBRM, whereas CUC1 and CUC3 are dependent on AtBRM alone.

Correct cotyledon development depends on the proper expression of all three CUC genes in a small group of cells, the cotyledon boundary, in the early heart stage embryo (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). SYD expression overlaps with that of the CUC genes; SYD is expressed throughout young embryos, including during the early heart stage when the cotyledon boundary becomes established (Wagner and Meyerowitz, 2002) (data not shown). AtBRM and SYD have a similar expression pattern throughout Arabidopsis development (Farrona et al., 2004; Wagner and Meyerowitz, 2002), although the spatial expression of AtBRM in the embryo is not known. Because both SYD and AtBRM regulate expression of the CUC genes in the cotyledon boundary, AtBRM as well as SYD is likely to be present in these cells. We therefore propose that the dependence of CUC1 expression on AtBRM alone (and not SYD) is likely to be due to functional differences in the primary sequence between the two ATPases rather than to a differential expression of the two ATPases.

The cotyledon fusion defects even in strong cuc2-1 atbrm-1 and cuc1-1 syd-2 mutants are not fully penetrant. In addition to cup-shaped seedlings, we frequently observed cotyledons fused at only one margin (i.e. heart-shaped seedlings) in cuc1-1 syd-2 and cuc atbrm-1 double mutants. In these heart-shaped seedlings, one side of each cotyledon blade is completely separated, whereas the other sides are fused together. We show that CUC promoter activity is exclusively reduced in the fused region in the embryo. The data suggest that absence of AtBRM or SYD in a sensitized genetic background leads to a stochastic loss of CUC gene transcription, resulting in cotyledon fusion. AtBRM and SYD may therefore be required for the maintenance of CUC expression in the boundary region. Similarly, SYD is required for the maintenance of WUS expression in the inflorescence SAM (Kwon et al., 2005), and the Trithorax group protein SNF2 ATPase Brahma is required for the maintenance of HOX gene expression in Drosophila (Tamkun et al., 1992). Alternatively, SYD and AtBRM may be required for the initiation of CUC expression in the cotyledon boundary.

Fig. 5. CUC::GUS expression in embryos. (A) Top view of the embryo apex. The cotyledon boundary is indicated in turquoise: C, cotyledon; EM, embryonic meristem. (B-D) Siliques of cuc2-1/cuc2-1 atbrm-1/+ (B-I) and cuc1-1/cuc1-1 syd-2/+ (J-O) plants were examined. Genotypes are indicated to the right. cuc2 comprises cuc2-1/cuc2-1+/+ and cuc2-1/cuc2-1 atbrm-1/+, whereas cuc1 comprises cuc1-1/cuc1-1+/+ and cuc1-1/cuc1-1 syd-2/+. Representative embryos expressing CUC1::GUS (B-D,G,J,M), CUC2::GUS (E,H,K,N) and CUC3::GUS (F,I,L,O) are shown. When viewed from the front (f), CUC::GUS expression in the cotyledon boundary will appear as a small circle of expression (C-F,J-L). When viewed from the side (s) a line of expression will be observed (B). Embryos were at the torpedo stage (B,C) or at the bent cotyledon stage (D-O). Bent cotyledon stage embryos with fused cotyledons present a side view (s), whereas non-fused embryos usually present a front (f) view. Orientation (s,f) is indicated in each panel. (Cuc1) the same cuc2-1 atbrm-1 embryo is shown from two sides: nonfused (left) and fused (right). (G-I) CUC::GUS expression missing in the presumptive cotyledon boundary in the double mutants is indicated by a yellow arrow. (N) CUC2::GUS expression in the boundary between the cotyledons is severely reduced in cuc1-1 syd-2. Faint residual CUC2::GUS expression was observed in the presumptive boundary between each cotyledon and the shoot apical meristem, consistent with the observed lack of shoot apical meristem defects in cuc1-1 syd-2 (Fig. 2C). Where applicable, red arrowheads indicate the end points of cotyledon fusions (C,G-I,M-O). Scale bars: 100 μm.

Regulation of CUC expression in the cotyledon boundary
Recently, several mechanisms were described that control spatial CUC expression as well as CUC levels (Aida and Tasaka, 2006). Polar auxin transport appears to be required for restriction of CUC
expression to the cotyledon boundary, and may also control CUC expression levels within the cotyledon boundary (Aida et al., 2002; Furutani et al., 2004). The microRNA miR164 family negatively regulates CUC1 and CUC2 expression levels: overexpression of miR164 in transgenic plants leads to a reduction in the mRNA levels of CUC1 and CUC2 (Baker et al., 2005; Laufs et al., 2004; Mallory et al., 2004), whereas mutants that affect miR164 accumulation result in increased CUC1 and CUC2 expression, and in an enlarged boundary domain (Baker et al., 2005; Laufs et al., 2004). One candidate positive regulator of CUC expression in the boundary region is the homeodomain-containing transcription factor STM (Aida et al., 1999; Aida et al., 2002; Kwon et al., 2005) (Fig. 6A,B). Strong stm-1 mutants exhibit fusion at the base of the cotyledon petiole, which is enhanced in double mutant combinations with cuc1, cuc2 and syd-2 (Aida et al., 1999; Kwon et al., 2005). stm-1 has overall reduced levels of CUC2, as well as improper spatial CUC1 and CUC2 expression (this study) (Aida et al., 1999; Kwon et al., 2005; Takada et al., 2001). In addition, STM appears to be required for maintaining CUC1 activity in pin1 mutants (Aida et al., 2002). We show here that the cotyledon fusion and molecular defects in stm-2 atbrm-1 and stm-2 syd-2 exceed those observed in the strong stm-1 allele, suggesting that AtBRM and SYD are not likely to act in the STM pathway (Fig. 6A,B).

It is currently not clear whether SYD and AtBRM act directly on CUC gene expression, or whether they control the expression of transcription factors required for CUC expression. Whatever the mechanism, our data indicate that AtBRM acts via a different trans-acting factor or a different cis-regulatory DNA element than does SYD, at least for the regulation of CUC1 expression (Fig. 6).

CUC gene expression and plant form

Our findings suggest that chromatin remodeling is important for proper transcription of the CUC genes in Arabidopsis and that the cis-regulatory elements required for this regulation are in the intergenic regions, because the CUC reporter constructs were dependent on AtBRM and SYD for proper expression. The mRNA levels of CUC1 and CUC2, but not CUC3, are also controlled by the miR164 family of microRNAs, which directs CUC message cleavage after binding to a complementary sequence in the last exon of CUC1 and CUC2 (Baker et al., 2005; Laufs et al., 2004; Mallory et al., 2004). The presence of independently mediated microRNA- and chromatin-based mechanisms for the regulation of CUC gene expression suggests that the precise level of expression of these genes is crucial for proper development (Baker et al., 2005; Laufs et al., 2004) (this study). It is tempting to speculate that, because CUC gene expression controls the presence and size of the boundary domain between primordia, it modulates the shape of leaves and flowers and thus contributes to the diversity of these structures found throughout the plant kingdom.

In support of this notion, independent gene duplication events are observed among the CUC genes in monocots and eudicots (Zimmermann and Werr, 2005). In the case of the eudicots, phylogenetic analysis shows that CUC1 and CUC2 are closely related members of the 105 member NAC domain family in Arabidopsis (Zimmermann and Werr, 2005). In two asterids (a closely related subclass of core eudicots), a single protein (NAM in petunia and CUP in snapdragon) is present, and organ boundary formation defects are observed in the single mutants in both species (Sower et al., 1996; Weir et al., 2004). This suggests that CUC1 and CUC2 are likely to have arisen from a relatively recent duplication event. By contrast, CUC3 appears to be conserved between monocots and eudicots (Zimmermann and Werr, 2005).

AtBRM and SYD also have direct orthologs in monocots (Su et al., 2006) (www.chromdb.org), indicating that AtBRM and SYD existed before the separation of these two classes of flowering plants. Thus, events that resulted in the requirement of SYD only for CUC2 expression may have occurred after duplication of the ancestral CUC gene. One possible scenario is that CUC1 selectively lost a regulatory element required for direct or indirect SYD-mediated activation of gene expression. By contrast, duplication of the ancestral CUC gene did not change microRNA-mediated regulation of CUC1 and CUC2 expression because the miR164 target site in the coding region is preserved in both CUC1 and CUC2, but is absent from CUC3 (Fig. 6C) (Baker et al., 2005; Laufs et al., 2004; Mallory et al., 2004). The independent regulation of CUC expression by chromatin remodeling and microRNA-mediated post-transcriptional control, together with recent CUC gene family expansion and reduction events, point to a possible role for spatial and quantitative regulation of CUC in generating variety in plant form.

Developmental specificity of SWI/SNF ATPases

SWI/SNF chromatin remodeling ATPases are transcriptional co-regulators that control the accessibility of the nucleosomal DNA to transcription factors and the transcriptional machinery (Cairns, 2005; Mohrmann and Verdier, 2005). Most multicellular eukaryotes have multiple SWI/SNF ATPases (Cairns, 2005; Mohrmann and Verdier, 2005; Tsukiyama, 2002) (www.chromdb.org). Despite their importance in normal development and differentiation and as tumor suppressors (Bultman et al., 2000; Bultman et al., 2005; de la Serna et al., 2006; Indra et al., 2005; Reisman et al., 2002; Sawa et al., 2000; Simone, 2006; Tamkun et al., 1992; Watanabe et al., 2006), it is currently not understood whether individual chromatin remodeling factors have redundant, overlapping or unique roles in the organism, as many SWI/SNF ATPases are embryo lethal (Bultman et al., 2000; Bultman
et al., 2005; Indra et al., 2005; Reyes et al., 1998; Sawa et al., 2000; Tamkun et al., 1992). The availability of viable null mutants of both SYD and AtBRM has allowed us to uncover specific, overlapping roles for the two ATPases in at least one developmental process in Arabidopsis, cotyledon separation. In addition, SYD appears to play a more specialized role than does AtBRM, a finding that is consistent with the more unique domain architecture of this SWI/SNF ATPase.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/16/3223/DC1

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