The *Arabidopsis thaliana* SNF2 homolog AtBRM controls shoot development and flowering

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Summary

Chromatin remodeling is essential for the reprogramming of transcription associated with development and cell differentiation. The SWI/SNF complex was the first chromatin remodeling complex characterized in yeast and *Drosophila*. In this work we have characterized an *Arabidopsis thaliana* homolog of Brahma, the ATPase of the *Drosophila* SWI/SNF complex. As its *Drosophila* counterpart, *Arabidopsis thaliana* BRAHMA (AtBRM) is a nuclear protein present in a high molecular mass complex. Furthermore, the N terminus of AtBRM interacts, in the two-hybrid system, with CHB4 (AtSWI3C), an *Arabidopsis* homolog of the yeast SWI/SNF complex subunit SWI3. The *AtBRM* gene is primarily expressed in meristems, organ primordia and tissues with active cell division. Silencing of the expression of the *AtBRM* gene by RNA interference demonstrated that AtBRM is required for vegetative and reproductive development. The *AtBRM* silenced plants exhibited a reduction in overall plant size with small and curled leafs, as well as a reduction in the size of the inflorescence meristem. In the absence of AtBRM, *Arabidopsis* flowers have small petals and stamens, immature anthers, homeotic transformations and reduced fertility. The *AtBRM* silenced plants flower earlier than wild-type plants both under inductive and non-inductive photoperiods. Furthermore, levels of *CO*, *FT* and *SOC1* transcripts were up-regulated under non-inductive conditions suggesting that AtBRM is a repressor of the photoperiod-dependent flowering pathway.

Key words: Chromatin, Transcription, CONSTANS, SWI/SNF, CHB4, *Arabidopsis thaliana*

Introduction

How specific patterns of gene expression are established and maintained during generations in precise cell lineages is an essential topic in developmental biology. Chromatin organization plays a crucial role in controlling gene expression and in the inheritance of transcription patterns by epigenetic mechanisms (Muller and Leutz, 2001). Therefore, there has been increasing interest in recent years in understanding how chromatin modifiers affect plant development (for reviews, see Berger and Gaudin, 2003; Habu et al., 2001; Li et al., 2002; Reyes and Grossniklaus, 2003; Reyes et al., 2002; Verbsky and Richards, 2001; Wagner, 2003). In *Drosophila*, the interplay between two antagonistic groups of chromatin factors, the Polycomb group (PcG) and the Trithorax group (TrxG), controls expression of homeotic genes (Simon and Tamkun, 2002). In PcG mutant embryos patterns of homeotic gene expression are initially established, but later in the development memory of transcriptional states is lost and homeotic genes are expressed outside their normal limits. Hence, PcG proteins maintain the repressed state in those segments where the target genes were initially silenced. TrxG proteins counteract the effect of the PcG proteins on homeotic gene expression (Kennison and Tamkun, 1988). Biochemical studies have shown that several TrxG proteins such as Osa, Moira, Snr1 and Brahma, co-purify in a nuclear complex termed the Brahma complex (Dingwall et al., 1995; Papoulas et al., 1998). A similar complex was originally identified in *Saccharomyces cerevisiae* and named the SWI/SNF complex (Cairns et al., 1994; Cote et al., 1994; Peterson et al., 1994), and later, also identified in mammals (Wang et al., 1996). SWI/SNF complexes are chromatin remodeling machines that use energy from ATP hydrolysis to destabilize interactions between histone octamers and DNA in the nucleosome. The mechanism by which SWI/SNF-like complexes reorganize nucleosomal interactions remains unknown. One of the proteins of the *Drosophila* complex, Brahma and its yeast counterpart SWI2/SNF2, belong to a conserved family of DNA-dependent ATPases termed the SNF2 family (Peterson and Herskowitz, 1992; Tamkun et al., 1992). A conserved domain responsible for the DNA-dependent ATPase activity, which shares homology with DNA helicases, characterizes proteins of this family, which are found in all examined eukaryotes. These proteins are the catalytic subunit of a plethora of ATP-dependent chromatin remodeling complexes. Besides the conserved ATPase domain, proteins of the SNF2 family have different amino-terminal and carboxyl-terminal domains that are involved in interaction with other proteins of the complexes, or with other chromatin proteins. These domains determine the subfamilies of the SNF2 family, such as the SWI2/SNF2, the ISWI, or the CHD subfamilies (Eisen

et al., 1995). All the identified SWI/SNF-related complexes contain an ATPase of the SWI2/SNF2 subfamily.

The *Arabidopsis* genome contains about 40 loci encoding putative proteins of the SNF2 family. Some of them have been shown to be essential for *Arabidopsis* development (reviewed by Reyes et al., 2002; Verbsky and Richards, 2001; Wagner, 2003). For example, PICKLE belongs to the CHD subfamily and is involved in the suppression of embryonic and meristematic characteristics during development (Eshed et al., 1999; Ogas et al., 1997; Ogas et al., 1999). DECREASE IN DNA METHYLATION1 (DDM1), is required for cytosine methylation and the histone H3 methylation patterns (Gendrel et al., 2002; Jeddeloh et al., 1999). PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 (PIE1) belongs to the Domino/SWR1 subfamily and controls flowering time by regulating the expression of *FLOWERING LOCUS C* (*FLC*) (Noh and Amasino, 2003). Finally, SPLAYED (SYD) belongs to the SWI2/SNF2 subfamily and has been shown to play different roles in apical meristem identity and carpel development (Wagner and Meyerowitz, 2002). We report that *Arabidopsis thaliana* BRAHMA (AtBRM), an *Arabidopsis* homolog of *Drosophila* Brahma, is a nuclear protein assembled in a high molecular mass complex, mostly expressed in meristems and proliferating tissue, and is required for normal vegetative and reproductive development.

Materials and methods

Plant material and growth conditions

Wild-type *Arabidopsis thaliana* (ecotype Columbia) plants were grown either in pots containing a mixture of compost and vermiculite (3:1) or aseptically in Petri dishes containing Murashige and Skoog medium supplemented with 1% (w/v) sucrose and 0.37% (w/v) Phytagel (Sigma). Plants were grown in a cabinet under long-day (16 hours light/8 hours dark) or short-day (10 hours light/14 hours dark) photoregimes at 23°C (day)/20°C (night), 70% relative humidity, and a light intensity of 130 μE m^{-2} s^{-1} supplied by white fluorescent lamps.

Cell extracts

Total cell extracts were carried out as described previously (Martinez-Garcia et al., 1999). For nuclear extracts, nuclei were isolated as described previously (Guilfoyle, 1995). For western blotting isolated nuclei were lysed in NIB [20 mM Tris-HCl (pH 7.2), 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol, 40% (v/v) glycerol (Guilfoyle, 1995)] supplemented with 1.5% (w/v) SDS. Total protein was determined by a modified Lowry procedure using ovoalbumin as the standard (Markwell et al., 1978). For size-exclusion chromatography nuclear proteins were extracted with 0.4 M $(NH_4)_2SO_4$ in NIB buffer. After 30 minutes at 4°C, nuclear debris was pelleted by centrifugation and proteins were precipitated by adding 0.3 g/ml of ammonium sulphate. The protein pellet was resuspended in NIB and loaded into a Superose 6 HR 10/30 column (Amersham) connected to a HPLC.

Generation of the cDNA corresponding to AtBRM

A partial *AtBRM* cDNA clone was obtained from ABRC (accession number H8A3T7). In addition, five partial overlapping cDNA fragments were generated by RT-PCR and cloned into pGEN-T. Primer sequences and details about cDNA reconstruction are available on request. The *AtBRM* cDNA was fully sequenced. EMBL Nucleotide Sequence Database accession number AJ703891.

Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed with the PROQUEST two-hybrid system (Invitrogen). A 2810 bp fragment of the *AtBRM* cDNA, encompassing amino acids 16 to 952 of the protein, was inserted in the pDBleu plasmid in phase with the GAL4 DNA binding domain (GBD-$AtBRM_{16\text{-}952}$). Full length cDNAs for *CHB4* (obtained from Kazusa DNA Research Institute, accession number AV524064) and *BSH* (provided by A. Jerzmanowski) were cloned into pPC86, in phase with the GAL4 activation domain (GAD-CHB4 and GAD-BSH). Interaction experiments were carried out in the yeast strain MaV203. β-galactosidase activity was determined using the colony lift assay and the liquid assay as recommended by the manufacturer's instructions.

AtBRM antibodies and western blotting

Two different anti-AtBRM rabbit polyclonal antibodies were raised against recombinant fusion proteins. To produce the AtBRMa antigen a fragment of the *AtBRM* cDNA encoding amino acids 1758-1920 was inserted into the pGEX4T plasmid in-frame with the glutathione S-transferase (GST). To produce the AtBRMb antigen, a fragment of the *AtBRM* cDNA encoding amino acids 2047-2187 was inserted into the pET24a plasmid in order to generate a 6× histidine-tagged version of the peptide. The fusion proteins were expressed in *Escherichia coli* and affinity purified on glutathione-Sepharose 4B (Amersham), and His-Bind resin matrix (Novagen), respectively. Purified AtBRMa and AtBRMb proteins were used to raise polyclonal antiserum in rabbit (α-AtBRMa and α-AtBRMb, respectively). For western blot analysis 10 μg of nuclear proteins were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was then blocked with PBS/0.5% (v/v) Tween 20/5% (w/v) fat-free milk power and incubated with the appropriate antisera (1/2000 dilution). Enhanced chemiluminescence (ECL) reagents (Amersham) were used for detection.

Gene expression analysis

RNA was isolated as described previously (Kalantidis et al., 2000) or by using the RNeasy Mini Kit (Qiagen). For northern blotting, 10 μg of total RNA were loaded per lane and electrophoresed in 1.2% agarose denaturing formaldehyde gels. Transfer to nylon membranes (Hybond N-plus; Amersham), prehybridization, hybridization and washes were performed as described in the Amersham instruction manual. Probes were labeled with [α-^{32}P]dCTP using the Ready To Go labeling kit. For semi-quantitative RT-PCR, 5 μg of total RNA were used to generate the first-strand cDNA by using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). PCR amplification was performed using 2 μl of a 20 μl of RT reaction. Specific primers were used for 20 amplification cycles and DNA products were detected by Southern blot hybridization. The number of PCR cycles chosen was shown to be in the linear range of the reaction in a separate experiment. Primer sequences and details about the probes used for northern and Southern experiments are available on request.

β-glucuronidase (GUS) activity was assayed according to the method of McConnell and Barton (McConnell and Barton, 1998). GUS activity was allowed to develop for 48 hours. For visualizing sectioned GUS stained samples, tissue was first GUS stained and then sectioned into resin. GUS-stained tissue was embedded in resin and sectioned according to standard procedures.

Microscopy

SEM and histological analysis was carried out as described previously (Siegfried et al., 1999; Emery et al., 2003).

Transformation vectors and construction of transgenic plants

To generate plants with reduced levels of expression of AtBRM, a 584 bp DNA fragment encompassing nucleotides 4558-5142 of the

AtBRM cDNA, was amplified by PCR using primers that added *Xho*I and *Kpn*I sites at the ends of one product and *Bam*HI and *Cla*I sites at the ends of the other product. These two amplification products were then directionally cloned into pHANNIBAL (Wesley et al., 2001) to generate pHANNIBAL-AtBRM. Then the *Not*I-*Not*I fragment of pHANNIBAL-AtBRM was introduced into the *Not*I site of the binary vector pART27 to generate pART27-AtBRM-RNAi.

To generate plants that expressed the GUS gene under the control of the *AtBRM* promoter and regulatory regions, two different DNA fragments containing 5′ upstream regions of the *AtBRM* gene were fused to the GUS gene. A 1371 bp fragment 5′ to the first exon of *AtBRM* (nucleotide 18870671 to 18872042) was cloned into pRITAI to generate pRITA-AtBRM-P. A 1707 fragment including the same region as in pRITA-AtBRM-P, plus the first exon and the first intron of *AtBRM* (nucleotide 18870671 to 18872375) was cloned into pRITAI to generate pRITA-AtBRM-PI. The *Not*I fragments of these plasmids were introduced into the binary vector pART27, to generate pAtBRM-P-GUS and pAtBRM-PI-GUS, respectively.

Arabidopsis was transformed by the dipping method of (Clough and Bent, 1998). Seeds from treated plants were collected and screened for kanamycin resistance. Transgenic plants identified in this generation were classified as T_1 plants.

Results

Identification of an *Arabidopsis* protein homolog of *Drosophila* Brahma

Several conserved domains have been described in ATPases of SWI/SNF complexes from different origins (Fig. 1). Domains 1 and 2 were originally defined in the amino-terminal part of the *Drosophila* Brahma protein and seem to be involved in protein-protein interactions (Tamkun et al., 1992). The AT hook is a non-specific DNA-binding domain (Bourachot et al., 1999). The bromodomain is present in several chromatin-associated proteins and is involved in the interaction with acetylated lysines (Dhalluin et al., 1999). Finally, poly-glutamine domains are also often found in the amino-terminal part of this subfamily of proteins. Analysis of the *Arabidopsis* genome provided evidence for four loci encoding proteins related to *Drosophila* Brahma: At2g46010-At2g46020, At5g19310, At3g06010 and the previously characterized *SPLAYED* (At2g28290) (Wagner and Meyerowitz, 2002). At2g46010 and At2g46020 have been annotated as two contiguous different loci, however the amino acid sequence deduced from At2g46010 and At2g46020 are related to the amino- and carboxyl-terminal halves of Brahma, respectively, suggesting that At2g46010 and At2g46020 constitute a single gene. This was confirmed by RT-PCR experiments using oligonucleotide pairs that span from the last exons of At2g46010 to the first exons of At2g46020 (data not shown). Fig. 1 shows the domain organization of the four closest *Arabidopsis* homologues of Brahma together with *Drosophila* Brahma and yeast SWI2/SNF2. Among the *Arabidopsis* proteins only that encoded by the At2g46010-At2g46020 locus possesses all the domains previously described for the *Drosophila* Brahma protein and for the proteins of the SWI2/SNF2 subgroup. While existence of a partial bromodomain motif in the SYD protein has been proposed (Wagner and Meyerowitz, 2002), our amino acid sequence analysis argues against this possibility. Therefore, we have designated the gene encoded by the At2g46010-At2g46020 locus as *Arabidopsis thaliana BRAHMA* (*AtBRM*). The complete sequence of the *AtBRM* cDNA has been obtained by sequencing the H8A3T7 EST and five partial overlapping cDNA fragments that were generated by RT-PCR.

AtBRM is associated to a high molecular mass complex in the nucleus

To investigate the subcellular distribution of the AtBRM protein, cytoplasmic and nuclear extracts were isolated and subjected to Western blotting using two different antibodies generated against two different parts of AtBRM (see Materials and methods). As shown in Fig. 2A, both antibodies recognized a protein of about 250 kDa, consistent with the deduced molecular mass of the protein encoded by the At2g46010-At2g46020 locus (245,317 Da). The AtBRM protein was only

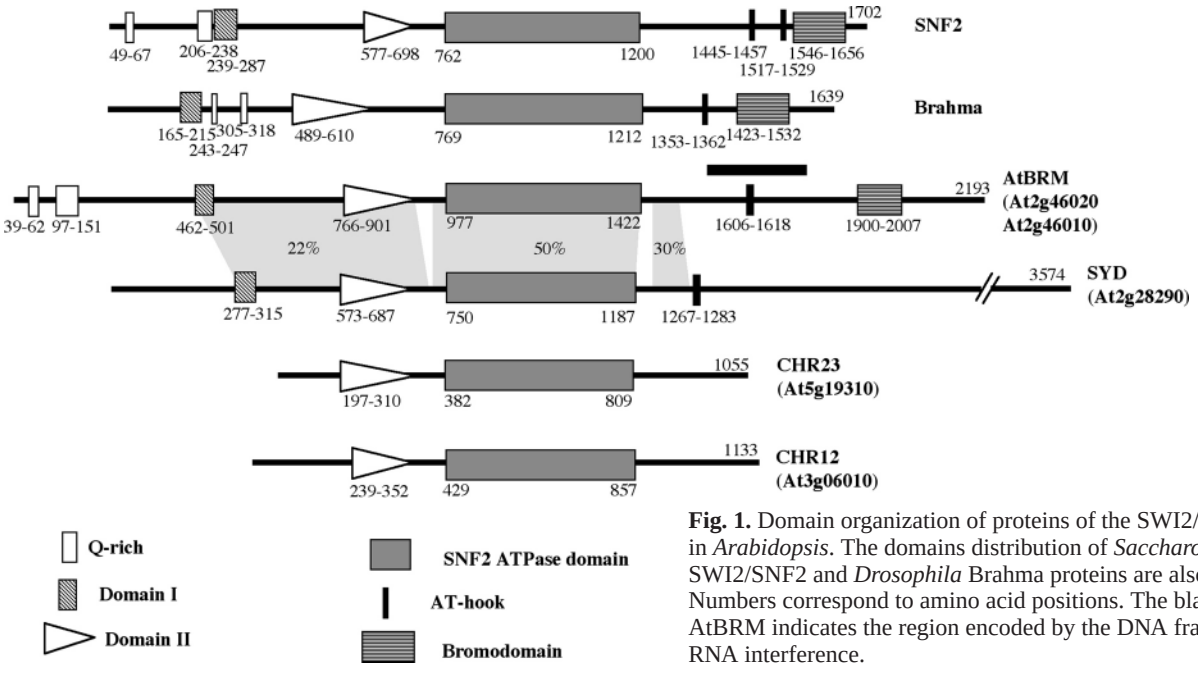

Fig. 1. Domain organization of proteins of the SWI2/SNF2 subfamily in *Arabidopsis*. The domains distribution of *Saccharomyces cerevisiae* SWI2/SNF2 and *Drosophila* Brahma proteins are also included. Numbers correspond to amino acid positions. The black bar above AtBRM indicates the region encoded by the DNA fragment used for RNA interference.

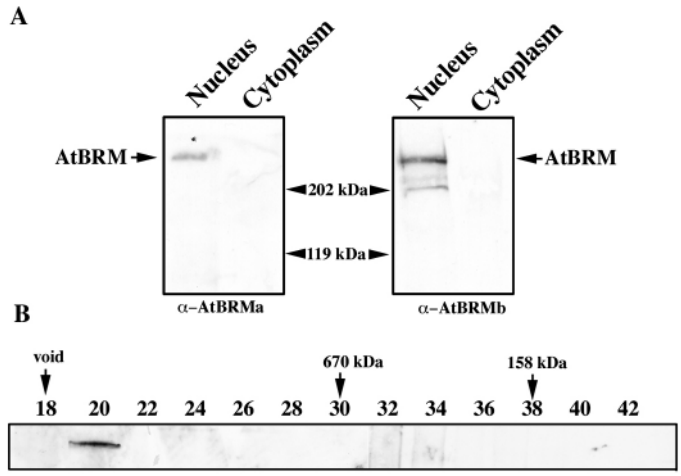

Fig. 2. AtBRM is a nuclear protein associated with a high molecular mass complex. (A) Nuclear and cytoplasmic proteins from inflorescence apices were subjected to western blotting using two different antisera against two different regions of AtBRM (α-AtBRMa and α-AtBRMb). (B) Gel filtration analysis of nuclear extracts of *Arabidopsis* calli. Fractions from chromatography through a Superose 6 HR column were separated by SDS-PAGE and examined by western blotting using α-AtBRMb antiserum. Fraction numbers are given across the top and arrows indicate elution position of molecular mass standards.

detected in nuclear extracts. Brahma and other ATPases of the SNF2 family are frequently associated with other proteins and fractionate as high molecular mass complexes in gel filtration chromatography. Nuclear proteins extracted from *Arabidopsis* callus were fractionated on a Superose 6 FPLC column, and the eluted fractions were assayed for AtBRM protein by western blotting (Fig. 2B). The apparent native molecular mass of the AtBRM protein was between 1 and 2 MDa, indicating that AtBRM is incorporated into a multi-protein complex in the nuclei of *Arabidopsis* cells.

AtBRM interacts with CHB4 (AtSWI3C)

We investigated whether AtBRM interacts with other *Arabidopsis* putative SWI/SNF subunits using the yeast two-hybrid system. CHB4 (AtSWI3C, At1g21700) is an *Arabidopsis* homolog of the SWI3 and MOIRA proteins from yeast and *Drosophila*, respectively (Sarnowski et al., 2002) (see Plant Chromatin Database: http://chromdb.org). SWI3 and MOIRA interact directly with the amino-terminal regions of SWI2/SNF2 and Brahma, respectively (Crosby et al., 1999; Treich et al., 1995). Therefore, we tested whether the amino terminal half of AtBRM interacts with the full length CHB4 protein. In these experiments the DNA binding partner contained AtBRM residues 16 to 952 fused to the GAL4 DNA-binding domain (GBD-AtBRM$_{16-952}$) while the full length CHB4 protein was fused to the GAL4 activation domain (GAD-CHB4). As shown in Table 1, the co-expression of both proteins in yeast activated the expression of a chromosomal *GAL1*::*lacZ* reporter gene, indicating that the amino terminus of AtBRM interacts with CHB4. Furthermore, the yeast strain co-expressing both fusion proteins was able to grow in selective medium without histidine, indicating the activation of a *GAL1::HIS3* reporter gene (data not shown). We next

Table 1. Interaction of AtBRM$_{16-952}$ with CHB4 in the yeast two-hybrid system

DNA-binding protein	Activation protein	β-Galactosidase activity (mU)*
GBD	GAD	40±4
GBD-AtBRM$_{16-952}$	GAD	56±12
GBD-AtBRM$_{16-952}$	GAD-CHB4	702±35
GBD	GAD-CHB4	71±10
GBD-AtBRM$_{16-952}$	GAD-BSH	47±6
GBD	GAD-BSH	20±10

*Numbers are means of three replicates ± standard deviation.

investigated whether BUSHY GROWTH (BSH) (Brzeski et al., 1999), the *Arabidopsis* homologue of the yeast SNF5 protein interacts with AtBRM. It has been shown that the amino terminal part of human BRM is essential to co-immunoprecipitate human SNF5 (Muchardt et al., 1995); however, a direct interaction between these two proteins has never been observed, suggesting that hBRM and hSNF5 interact through an intermediary protein in the SWI/SNF complex. Consistent with these data, GAD-BSH was unable to interact with GBD-AtBRM$_{16-952}$ in our yeast two-hybrid assays.

AtBRM is primarily expressed in meristems and proliferating tissues

AtBRM mRNA was detected by northern blotting using total RNA from inflorescences, calli, and cultured cells and, to a lesser extent, from leaves, but not from siliques, roots or seedlings (Fig. 3A). However, *AtBRM* mRNA was also detected in these three tissues by RT-PCR (Fig. 3A, bottom panel), indicating that, albeit at a low level, *AtBRM* is also expressed in these tissues. Similarly, high levels of AtBRM protein were detected by western blotting in nuclear extracts of inflorescences, calli, cultured cells and leaves, while low levels were detected in extracts from siliques, roots and seedlings (Fig. 3B).

The spatial expression pattern of *AtBRM* was studied using transgenic lines that express the β-glucuronidase reporter gene (*GUS*) under the control of the *AtBRM* promoter and regulatory sequences. Two different constructs were generated. The first construct, pAtBRM-P-GUS contained a 1371 bp region upstream of the start codon of *AtBRM* fussed to the *GUS* gene. The second construct, pAtBRM-PI-GUS contained a 1707 fragment including the same region as in pAtBRM-P-GUS in addition to the first exon and the first intron of *AtBRM* fused to the *GUS* gene. Ten independent transgenic lines were analyzed for each construct. None of the ten lines containing the pAtBRM-P-GUS construct expressed GUS activity, however eight out of the ten lines transformed with the pAtBRM-PI-GUS construct showed a characteristic *GUS* expression pattern, indicating that the first exon and/or the first intron of the *AtBRM* gene contain elements required for its expression. GUS activity was observed in the vegetative shoot apical meristem (SAM) and in the root meristem of primary and secondary roots but not in lateral root primordia (Fig. 3C,E). GUS signal was also observed in young leaves and young floral buds, whereas signals diminished as the organs matured (Fig. 3F,G,H). In developing leaves, signal was mostly visible in the vascular tissue (Fig. 3D). In flowers the *AtBRM*

promoter was especially active in petals, stamens filaments, anthers and carpels (Fig. 3H). RNA in situ hybridization experiments were attempted unsuccessfully, probably because the low level of expression of the *AtBRM* gene.

AtBRM is required for normal vegetative and reproductive development

In order to investigate the function of AtBRM during *Arabidopsis* development transgenic lines with reduced levels of *AtBRM* expression were generated by RNA interference using the pHANNIBAL plasmid (Wesley et al., 2001). The cDNA fragment used for RNAi encompasses nucleotides 4558-5142 of the *AtBRM* cDNA sequence (encoding amino acids 1520-1713 of AtBRM protein; see Fig. 1). This DNA region shows no significant identity with the corresponding regions of the At5g19310, At3g06010 and *SYD* cDNAs, the

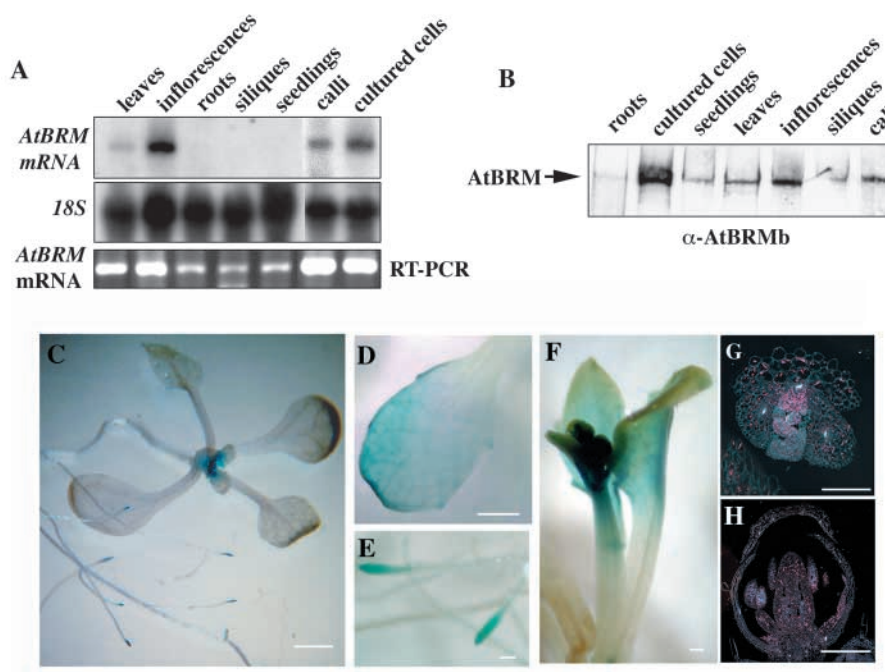

Fig. 3. *AtBRM* expression pattern. (A) *AtBRM* mRNA expression in different tissues. RNA was isolated from adult plants grown in long days. Total RNA blots were hybridized first with an *AtBRM* probe and then reprobed with 18S ribosomal DNA (18S) as a loading control. Level of *AtBRM* mRNA was also determined by RT-PCR (bottom panel). (B) AtBRM protein expression in different tissues. 10 μg of nuclear protein were separated by SDS-PAGE, and subjected to western blotting using α-AtBRMb antiserum. (C-H) Histochemical GUS staining of transgenic *Arabidopsis* plants containing an *AtBRM* promoter-first intron-GUS fusion (GUS staining is blue in whole mount, and pink when viewed with dark-field optics). (C) Ten-day-old whole seedling. Bar, 1 mm. (D) Magnification of a young leaf, showing staining of the vascular tissue. Bar, 1 mm. (E) Magnification of root tips of seedlings. Scale bar: 0.1 mm. (F) Inflorescence meristem. Scale bar: 0.1 mm. (G) Cross section of a vegetative shoot apical meristem, showing first leaves, leaf primordia and cotyledons. (H) Longitudinal section of floral bud at stage 12. Scale bar: 0.25 mm.

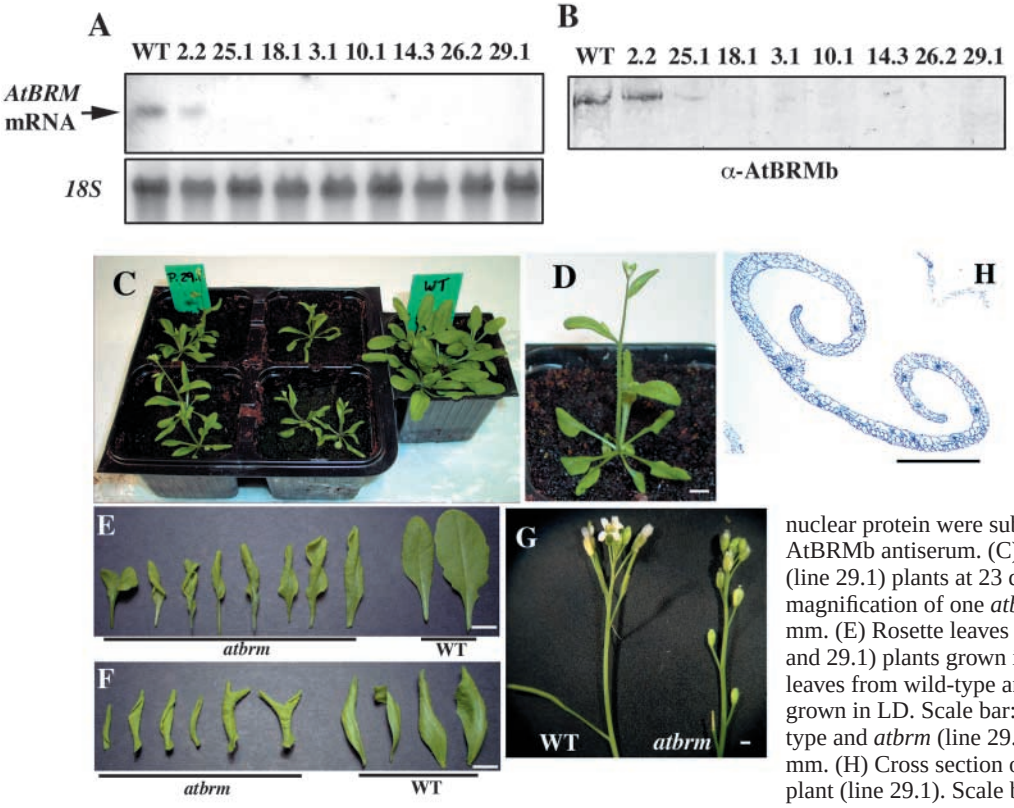

Fig. 4. Phenotypes of *AtBRM*-silenced plants (*atbrm*). (A) Levels of *AtBRM* mRNA in different kanamycin-resistant transgenic lines transformed with pART27-AtBRM-RNAi. Total RNA was isolated from inflorescence apices, blotted and hybridized with an *AtBRM* probe. The blot was stripped and rehybridized with an 18S ribosomal probe (18S) as a loading control. (B) Levels of AtBRM protein in *AtBRM*-silenced plants. 20 μg of nuclear protein were subjected to western blotting using α-AtBRMb antiserum. (C) Wild-type (Columbia) and *atbrm* (line 29.1) plants at 23 days of growth in LD. (D) Higher magnification of one *atbrm* plant shown in C. Scale bar: 5 mm. (E) Rosette leaves from wild-type and *atbrm* (lines 10.1 and 29.1) plants grown in LD. Scale bar: 1 cm. (F) Cauline leaves from wild-type and *atbrm* plants (lines 10.1 and 29.1) grown in LD. Scale bar: 1 cm. (G) Inflorescences from wild type and *atbrm* (line 29.1) plants grown in SDs. Scale bar: 1 mm. (H) Cross section of curled rosette leave from a *atbrm* plant (line 29.1). Scale bar: 1 mm.

closest *Arabidopsis* homologs of *AtBRM*. Among ten kanamycin-resistant transgenic lines analyzed eight showed different degrees of reduction in the level of expression of *AtBRM*, as evidenced by northern and western blotting experiments (Fig. 4A,B). Reduction of the AtBRM level was stable after several generations and similar in homozygous and heterozygous lines. Line 2.2 plants displayed a small reduction in the level of the *AtBRM* transcript, but levels of the AtBRM protein were not significantly affected. Line 2.2 plants displayed a wild-type (WT) phenotype. Lines 18.1, 3.1, 10.1, 14.3, 26.2 and 29.1, did not show detectable levels of either *AtBRM* transcript or protein and all exhibited a similar and characteristic phenotype (*atbrm* plants). Plants of the 25.1 line showed a strong reduction in the level of the *AtBRM* transcript, but a detectable amount of AtBRM protein (Fig. 4B) and had phenotypic characteristics intermediate between wild type and more completely silenced lines. In order to rule out the possibility that other related genes were affected by the RNA interference mechanism, expression of At5g19310, At3g06010 and SYD was analyzed by RT-PCR. Transcript levels of these three genes were not significantly altered in the *atbrm* plants in comparison with wild-type plants (data not shown). *AtBRM*-silenced plants exhibited a decrease in overall size with reduced stems and leaves (Fig. 4C,D). Rosette and cauline leaves of plants grown under long day (LD) conditions were strongly curled downwards and at the same time rolled in a spiral fashion (Fig. 4E,F,H). Leaves with a duplicated central vein were occasionally observed (Fig. 4E,F). Scanning electron microscopy revealed that abaxial and adaxial leaf epidermal cells of *atbrm* plants did not present obvious morphological abnormalities (data not shown). Root morphology, growth rate or number of secondary roots were not altered in *AtBRM*-silenced plants (data not shown).

The *AtBRM*-silenced plants also exhibited defects in floral organ size, number and identity. This phenotype was more dramatic under short day (SD) conditions than under LD conditions. A quantitative description of the flower phenotype of WT and *atbrm* lines 10.1 and 29.1 under SD conditions is presented in Table 2. Similar results were also observed in lines 18.1, 3.1, 14.3, 26.2. Flower anthesis was severely reduced (Fig. 4G). Most flowers had short petals and stamens (Fig. 5A-D) as well as short pedicels (Fig. 4G). The stamens exhibited several types of abnormalities: anther dehiscence was severely reduced; cells in the stamen filaments were always shorter than wild-type filament cells (Fig. 5G,H); occasionally, fused filaments or anthers with three locules were observed (Fig. 5D,E). Often a reduction in the number of stamens from six to four or five, was also observed (Fig. 5D). In about 35% of the flowers the gynoecium was bent, probably because of the failure of the flower to open after stage 12 (Fig. 5C). In the most dramatic cases flowers did not progress developmentally beyond stages 11 or 12, anthers did not open, and stigmatic papillae and style were not formed in the gynoecium (Fig. 5F). About 25% of flowers developed petals with patches of green tissue. These patches presented epidermal cells characteristic of sepals, including stomata (Fig. 5I-K). Flowers with these characteristics were unable to produce siliques. However, these flowers developed fruits after manual pollination with pollen from wild-type plants suggesting that the absence of silique development could be due to the defects in stamen development. Interestingly, *AtBRM*-silenced flowers showed a phenotypic gradient: early-arising basipetal flowers had shorter petals and stamens than later-arising acropetal flowers. For example, in line 29.1 petal and stamen length of early-arising flowers were 1.26±0.09 and 0.88±0.29 mm, respectively, in contrast to the 1.96±0.22 and 1.91±0.09 mm of petals and stamens of later-arising flowers. Length of wild-type petals and stamens was 2.99±0.16 and 2.44±0.38 mm, respectively. Other flower phenotypes such as reduction in flower anthesis and anther dehiscence also ocurred in a basipetal to acropetal gradient. Accordingly, later-arising flowers were able to produce small siliques with a limited number of seeds. Thus, late-arising siliques produce 8.44±3.58 seeds per silique in *atbrm* 29.1 plants versus 55.71±9.39 seeds per silique in WT plants.

There were fewer flower buds in the inflorescence of *atbrm* plants compared with those of wild-type plants (Fig. 6A). This effect was more dramatic at the later stages of the life of the plant. Interestingly, this was accompanied with a progressive reduction in the size of the inflorescence meristem. The mean number of flowers at stages 1-3 produced by a wild-type inflorescence meristem is about six, irrespective of the age of the meristem (see Table 2). However, young *atbrm* inflorescence meristems (23 days

Table 2. Phenotypic characteristics of *AtBRM* silenced flowers and inflorescences

Variable	WT (*n*=10)	10.1 (*n*=34)	29.1 (*n*=30)
Flower pedicel length (mm)*	4.75±0.63	2.41±0.56	2.47±0.80
Number of stamens*	5.90±0.32	4.94±0.74	4.83±0.65
Long stamens length (mm)*	2.44±0.38	0.69±0.29	0.88±0.28
Number of nondehiscent stamens per flower*	0	4.94±0.74	4.63±1.10
Flowers with fused stamen filaments*	0%	20.58%	23.30%
Flowers with curved gynoecium*	0%	35.29%	36.67%
Flowers with immature gynoecium*	0%	11.76%	16.67%
Petal length (mm)*	2.99±0.16	1.31±0.09	1.26±0.35
Flowers containing petals with sepalloid tissue*	0%	23.52%	26.60%
Flowers at stages 1-3 at day 23†	6.33±0.58 (*n*=3)	4.33±0.58 (*n*=3)	3.68±0.58 (*n*=3)
Flowers at stages 1-3 at day 28†	6.00±0.00 (*n*=3)	3.33±0.58 (*n*=3)	3.00±0.00 (*n*=3)
Flowers at stages 1-3 at day 33†	6.00±1.00 (*n*=3)	3.33±0.58 (*n*=3)	2.33±0.58 (*n*=3)
Flowers at stages 1-3 at day 38†	5.68±0.58 (*n*=3)	2.68±0.58 (*n*=3)	2.33±0.58 (*n*=3)

*Scored flowers were selected between the first eight arising flowers of wild-type, 10.1 and 29.1 plants grown under SD conditions. Numbers are means ± standard deviation.

†Number of flowers at stages 1-3 per primary inflorescences were counted. Wild-type, 10.1 and 29.1 plants were grown under LD photoperiod. Three primary inflorescences per day and per line were analyzed. Numbers are means ± standard deviation.

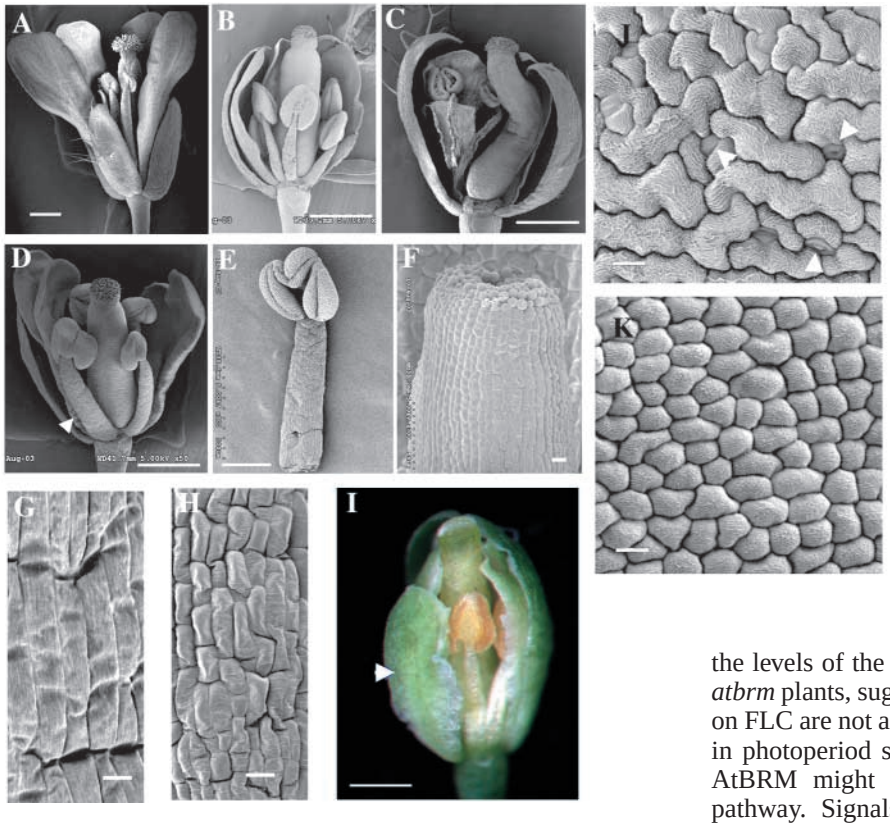

Fig. 5. Flower phenotypes of *AtBRM*-silenced plants. In all cases plants were grown under SD conditions. (A-D) Scanning electron micrographs of mature wild-type (A) and *atbrm* (B-D) flowers. Scale bar: 0.5 mm. The mutant flowers have a variety of phenotypes: (B) short petals and stamens, (C) bent carpel, (D) four stamens. The arrowhead in D indicates two fused stamens. (E) Higher magnification of fused stamens shown in D. Scale bar: 250 μm. (F) Gynoecium tip from an *atbrm* plant, stigma and style are absent. Scale bar: 10 μm. (G,H) Stamen filament cells from mature wild-type (G) and *atbrm* (H) flowers. Scale bar: 10 μm. (I) Sepaloid petal in an *atbrm* flower. Scale bar: 0.5 mm. (J) Abaxial cells from the sepaloid petal shown in I. Arrowheads indicate stomatal cells. Scale bar: 10 μm. (K) Abaxial cells from a wild-type petal. Scale bar: 10 μm. The *atbrm* plants belong to line 10.1 in all cases except C, which is line 29.1.

after sowing) produced an average of about four stage 1-3 flowers, while older inflorescence meristems (38 days after sowing) produced an average of about 2.5 stage 1-3 flowers (Fig. 6B, Table 2). The size of floral meristems was not significantly affected.

AtBRM controls transition from vegetative to reproductive development

Next we analyzed the role of *AtBRM* in the floral transition. Flowering time is often assayed as the total number of leaves at anthesis of the first floral bud. However, since *atbrm* plants presented anthesis problems we decided to measure the floral transition time by the number of days from sowing to bolting or by the number of rosette leaves formed at bolting. Fig. 7A and Table 3 show that *atbrm* plants flowered earlier than wild-type Columbia plants, both in terms of numbers of days to bolting and number of rosette leaves. Early bolting was observed both under LD and under SD conditions. However, although *atbrm* plants exhibit a decrease in photoperiod sensitivity, they are not photoperiod insensitive since they flower earlier under LD than under SD photoregimen.

To determine the cause of the early flowering phenotype, transcript levels of several genes controlling flowering time were monitored at 10, 13 and 16 days after germination, when the transition to reproductive development is occurring. Experiments were carried out under SD conditions, where greater differences in flowering time were observed between wild-type and *atbrm* plants. Signals from the autonomous, and the vernalization-dependent flowering pathways govern the expression of the flowering repressor gene *FLC* (Michaels and Amasino, 1999; Mouradov et al., 2002). As shown in Fig. 7B, the levels of the *FLC* transcript were similar in wild-type and *atbrm* plants, suggesting that flowering pathways that converge on FLC are not altered in the absence of AtBRM. The decrease in photoperiod sensitivity of the *atbrm* plants suggested that AtBRM might affect the photoperiod-dependent flowering pathway. Signals from the photoperiod-dependent pathway control the expression of CONSTANS (CO), a putative transcription factor that is required to promote flowering under LD but not under SD conditions (Putterill et al., 1995). Under inductive conditions the abundance of *CO* transcripts increases during the last part of the light period and shows the highest level between the onset of the night and dawn (Suarez-Lopez et al., 2001). Expression levels of *CO* mRNA were analyzed 9 hours after dawn, in wild-type and *atbrm* plants grown under SD conditions (10 hours light/14 hours dark). Under these conditions, *CO* mRNA levels were three- to fourfold higher in *atbrm* plants than in wild-type plants (Fig. 7C). It has been shown that CO promotes flowering by activating the expression of the *FLOWERING LOCUS T (FT)* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)* genes (Samach et al., 2000). Therefore, we checked whether expression of these two genes was also altered in the absence of AtBRM. As shown in Fig. 7D,E, levels of the *FT* and *SOC1* transcripts were also up-regulated in the *atbrm* plants with respect to wild-type plants.

Discussion

Cell differentiation and development require extensive reprogramming of transcription patterns. Studies during the last ten years have documented the importance of chromatin remodeling associated with changes in transcription patterns in eukaryotes. The first ATP-dependent chromatin remodeling machine characterized in eukaryotes was the SWI/SNF complex with focus on its enzymatic motor subunit SWI2/SNF2 in yeast or Brahma in *Drosophila* (Peterson and Herskowitz, 1992; Tamkun et al., 1992). In this work we report the characterization of the closest *Arabidopsis* homolog of Brahma. AtBRM is the only *Arabidopsis* SNF2-like ATPase that has a sequence related to the bromodomain, the most

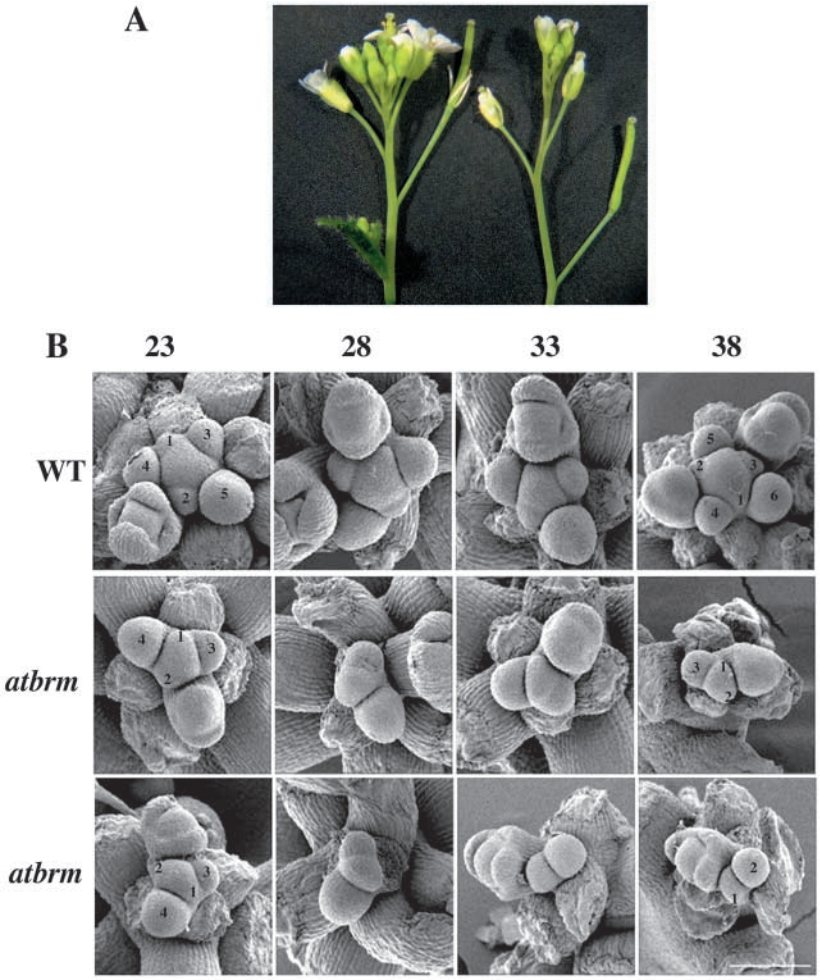

Fig. 6. Inflorescence meristem in *atbrm* plants. (A) Wild type (left) and *atbrm* line 29.1 (right) inflorescences. (B) Wild-type and *atbrm* line 29.1 inflorescence meristems at days 23, 28, 33 and 38. Stage 1-3 floral buds are numbered from youngest to oldest, in some of the micrographs. Scale bar: 100 μm.

characteristic domain of the ATPases associated with the SWI/SNF complexes in many species ranging from yeast to human. We show that AtBRM is assembled in a high molecular mass complex in *Arabidopsis* nuclei and that AtBRM interacts with CHB4, an homolog of the yeast and *Drosophila* SWI/SNF subunit SWI3/MOIRA. Therefore, our data suggest that AtBRM might form part of a putative *Arabidopsis* SWI/SNF-like complex.

AtBRM is strongly expressed in meristems, young organs and tissue composed of rapidly dividing cells. A similar expression pattern has been described for other members of the SNF2 family such as SYD, PKL and PIE1, consistent with the putative role of these proteins in chromatin remodeling associated with developmental transcriptional reprogramming (Eshed et al., 1999; Noh and Amasino, 2003; Wagner and Meyerowitz, 2002). Consistent with this pattern of expression we show that AtBRM is required for proper timing of the vegetative to reproductive phase transition in *Arabidopsis*. Thus, the reduction of AtBRM activity by RNA interference provokes an early flowering phenotype, especially under non-inductive photoperiods. We show that transcript levels of *CO*, *FT* and *SOC1* are up-regulated in *atbrm* plants indicating the constitutive induction of the photoperiod-dependent flowering pathway. While there is no evidence of direct control of transcription of *CO* or *FT* by AtBRM, it is worth noting the overlapping spatial patterns of expression of the three genes in developing leaf vascular tissue (Fig. 3D) (Takada and Goto, 2003). A similar early flowering phenotype, accompanied by ectopic expression of *FT* and to a lesser extend *CO*, has been described for the *tfl2* mutant (Gaudin et al., 2001; Kotake et al., 2003; Larsson et al., 1998). In addition, both *tfl2* and *atbrm* plants also have small epinastic leaves. *TFL2*, also called *LHP1*, encodes a protein homologous to the metazoan HP1 protein (Heterochromatin protein 1). HP1-like proteins contain a chromodomain involved in the molecular recognition of methylated lysine 9 of histone H3 which is a hallmark of silenced chromatin. An interaction between one of the human HP1 homologs and BRG1, the ATPase of the human SWI/SNF complex has been reported (Nielsen et al., 2002). The partially common phenotypes of *tfl2* and *atbrm* plants and the similar expression patterns (Fig. 3) (Kotake et al., 2003; Takada and Goto, 2003) may suggest a cooperation between TFL2 and AtBRM in the coordinate regulation of a number of developmental genes in *Arabidopsis*.

The *atbrm* flowers show a striking similarity to flowers of

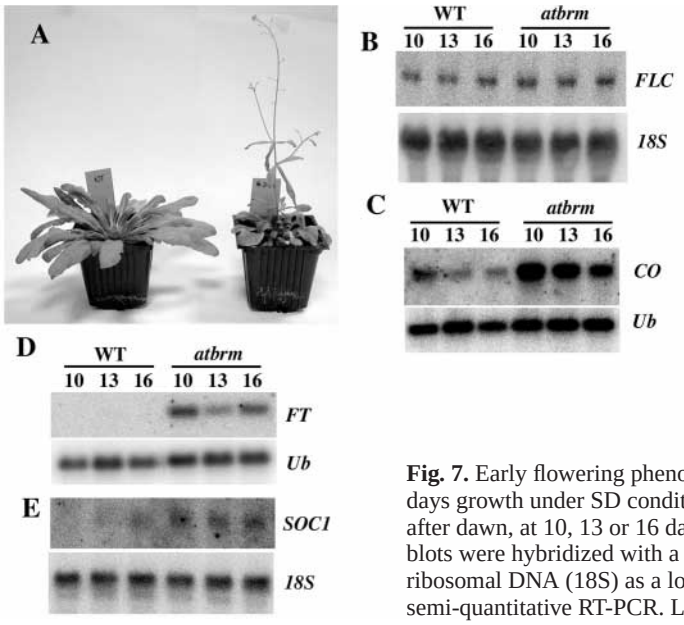

Fig. 7. Early flowering phenotype of *atbrm* plants (line 29.1). (A) Wild-type and *atbrm* plants at 40 days growth under SD conditions. (B-E) RNA was isolated from total seedlings, collected 9 hours after dawn, at 10, 13 or 16 days of growth under SD conditions (10 hours light/14 hours dark). RNA blots were hybridized with a *FLC*- (B) or with a *SOC1* (E)-specific probe and reprobed with 18S ribosomal DNA (18S) as a loading control. Levels of *CO* (C) and *FT* (D) mRNA were determined by semi-quantitative RT-PCR. Levels of the ubiquitin mRNA were also tested by RT-PCR as control.

Table 3. Bolting time phenotype of wild-type (Columbia) and *AtBRM* silenced plants

Line	LD (*n*>25*)	SD (*n*>10)	LL(*n*>15)§
Columbia	13.2±1.4† (23.7± 0.9)‡	45.0±6.0 (59.5±3.4)	12.1±1.6 (18.9±0.9)
10.1	6.9±1.1 (18.5±1.5)	14.1±2.9 (28.5±3.6)	7.8±1.3 (17.5±1.0)
14.3	6.8±1.5 (18.5±2.1)	13.5±2.0 (29.7±3.9)	7.9±1.3 (17.6±0.9)
26.2	6.5±1.3 (18.0±1.8)	12.9±1.0 (27.7±2.4)	n.d.¶
29.1	6.7±1.4 (18.4±2.8)	14.2±2.3 (28.7±2.1)	7.9±0.8 (17.8±1.0)

**n*, number of plants analyzed.
†Number of rosette leaves at bolting±standard deviation.
‡Number of days from sowing to bolting±standard deviation.
§LL, continuous light.
¶n.d., not determined.

the recently reported *gcn5-1* and *ada2b-1* mutant plants: they all exhibit small stamens and petals, defects in the elongation of the stamen filament and reduced fertility. The *Arabidopsis* GCN5 gene encodes a histone acetyltransferase homologous to the yeast GCN5 protein (Vlachonasios et al., 2003). In yeast, GCN5 and ADA2 form a transcriptional adaptor complex called SAGA which is able to acetylate histone H3 and H4 (Grant et al., 1997). Genetic interaction between components of SAGA and the SWI/SNF complexes suggests a functional link between them in yeast (Pollard and Peterson, 1997; Roberts and Winston, 1997). Recently, Hassan et al. (Hassan et al., 2002), have shown in yeast, that stable promoter occupancy by the SWI/SNF complex requires the acetylation of the chromatin template by the SAGA complex and that the acetylated-lysine binding activity of the bromodomain of SWI2/SNF2 is required in this process (Hassan et al., 2002). All these data indicate that the SWI/SNF and the SAGA complexes cooperate in the expression of a number of genes. Thus, expression of some genes in yeast requires the action of both the SAGA and the SWI/SNF complexes, while expression of other genes requires either one or the other complex (Holstege et al., 1998). This is consistent with our data in *Arabidopsis* where the *atbrm*, the *gcn5-1* and the *ada2b-1* plant phenotypes are similar in some aspects (floral morphology) but not in other aspects.

The most dramatic floral phenotypes of the *atbrm* plants affect the second and third floral whorls, and include small petals and stamens, non-dehiscent anthers and homeotic transformations (sepaloid petals). RT-PCR experiments showed that expression of the class B homeotic genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) is not significantly altered in *atbrm* plants (data not shown). One possibility would be that AtBRM cooperates with the AP3/PI heterodimer to control gene expression. The best characterized immediate target of AP3/PI is the *NAP* gene (*NAC-LIKE, ACTIVATED BY AP3/PI*) (Sablowski and Meyerowitz, 1998). Interestingly, both sense and antisense *35S::NAP* plants have flowers with small petals and stamens, resulting essentially from a defect in cell elongation, which is strikingly similar to the *atbrm* flowers phenotype. However, levels of *NAP* transcript determined by RT-PCR were not altered in the *atbrm* plants (data not shown), suggesting that AtBRM does not cooperate with AP3/PI to control NAP expression. Since NAP is also a transcription factor, an alternative scenario is that AtBRM and NAP cooperate to control the expression of NAP-regulated genes.

Flowers of *AtBRM*-silenced plant display a basipetal to acropetal phenotypic gradient. Thus, early arising flowers present a more dramatic phenotype than late arising flowers, suggesting that there is a progressive acropetal decrease in the requirement for AtBRM activity for flower development. Alternatively, AtBRM might control the expression of a protein that senses a signal distributed in an apical-basal gradient. Interestingly, *gcn5-1*, *ada2b-1* and *35S::antiNAP* inflorescences also display a phenotypic gradient: early-arising flowers have shorter petals and stamens than late-arising flowers, which again link the phenotype of these mutants with the *atbrm* plants (Sablowski and Meyerowitz, 1998; Vlachonasios et al., 2003).

The closest homologue of *AtBRM* in *Arabidopsis* is *SPLAYED (SYD)* (Wagner and Meyerowitz, 2002) (Fig. 1). The *syd* and *atbrm* plants have common and distinct phenotypic characteristics. The absence of SYD provokes a precocious transition from inflorescence to flower formation both under LD and SD, with a reduction in the number of secondary inflorescences that is not observed in the *atbrm* plants. Both AtBRM and SYD act as repressors of the phase transition in non-inductive conditions but *atbrm* plants also exhibit an early flowering phenotype under LD conditions. The general architecture of *syd* plants is similar to that of *atbrm* plants, with short stature, reduced leaf size and curled leaves. Interestingly, *syd* rosette leaves are curled upwards in contrast to the downward curling in *atbrm* plants. The closed *atbrm* flowers contrast with the characteristic splayed morphology of *syd* sepals, as a result of the outward bending of the pointed sepal tips. *syd* mutants also show reduced anther dehiscence, as do *atbrm* plants, but the size of stamens and petals is not altered. Therefore, it is possible that certain functional redundancy exists between these ATPases, however, a large number of functions of both proteins seem to be specific, consistent with most of the amino acid sequence identity between these proteins being concentrated in the ATPase domains, with the amino- and carboxy-terminal parts of the proteins very divergent or not related at all (see Fig. 1). As with AtBRM, SYD belongs to the SWI2/SNF2 subfamily of SNF2-like ATPases. SYD is probably also assembled in a SWI/SNF-like complex, different from the AtBRM-containing SWI/SNF complexes. The presence of a bromodomain in AtBRM may address the AtBRM-containing SWI/SNF complexes to a specific subset of genes different from that controlled by SYD.

AtBRM-silenced plants have a small inflorescence meristem and a concomitant decrease in the number of floral buds. A positive correlation between the size of the SAM and the number of flowers has been well established in *Arabidopsis* (e.g. Fletcher, 2001; Fletcher et al., 1999). Mutations in genes

that control SAM stem cell number, identity and differentiation, such as *CLAVATA1-3*, *WUSCHEL* and *SHOOT MERISTEMLESS*, severely affect the size of the SAM. Therefore, one possibility is that AtBRM modulates the expression of some of these genes. Alternatively, AtBRM may positively regulate the progression of the cell cycle, not only in SAM stem cells but also in differentiated cells. This more general effect is consistent with the small size of most organs of AtBRM-silenced plants. A role of the SWI/SNF complex in controlling cell cycle genes has been demonstrated in yeast and human (Krebs et al., 2000; Muchardt and Yaniv, 2001).

Plants with reduced levels of expression of other putative *Arabidopsis* SWI/SNF subunits have been reported (Brzeski et al., 1999; Zhou et al., 2003). For example, CHB2 encodes one of the four *Arabidopsis* homologues of the SWI3/MOIRA proteins (Sarnowski et al., 2002). As with *AtBRM*-silenced plants, *CHB2*-silenced plants have downward curled leaves (Zhou et al., 2003). However, in contrast to the *atbrm* plants, *CHB2*-silenced plants have abnormal cotyledons and roots, and delayed flowering. We have shown that AtBRM interacts with CHB4 in yeast two-hybrid experiments. However, it is presently unknown whether AtBRM might interact with CHB2 or other *Arabidopsis* SWI3/MOIRA-like proteins. The existence of small gene families for some of the putative SWI/SNF subunits in *Arabidopsis* (see Plant Chromatin Database: http://chromdb.org) suggests the existence of various types of SWI/SNF-like complexes with different subunit composition, and different functions. Therefore, it is not surprising that the phenotypes of plants with reduced levels of different putative SWI/SNF subunits differ.

A large number of chromatin-associated proteins, such as TFL2, (Gaudin et al., 2001; Kotake et al., 2003; Larsson et al., 1998), SYD (Wagner and Meyerowitz, 2002), PIE1 (Noh and Amasino, 2003), CLF (Goodrich et al., 1997), EBS (Pineiro et al., 2003), EMF2 (Chen et al., 1997), FIE (Kinoshita et al., 2001) and now AtBRM, seem to be involved in the repression of meristem phase transition. The switch from vegetative to reproductive growth is one of the most critical transitions in the life cycle of plants. How massive chromatin remodeling occurs in the SAM during this developmental transition and how this is controlled by environmental factors are some of the most interesting challenges for plant development research in the future.

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