AtMBD9 modulates Arabidopsis development through the dual epigenetic pathways of DNA methylation and histone acetylation

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SUMMARY

Mutations within the Arabidopsis METHYL-CpG BINDING DOMAIN 9 gene (AtMBD9) cause pleotropic phenotypes including early flowering and multiple lateral branches. Early flowering was previously attributed to the repression of flowering locus C (FLC) due to a reduction in histone acetylation. However, the reasons for other phenotypic variations remained obscure. Recent studies suggest an important functional correlation between DNA methylation and histone modifications. By investigating this relationship, we found that the global genomic DNA of atmbd9 was over-methylated, including the FLC gene region. Recombinant AtMBD9 does not have detectable DNA demethylation activity in vitro, but instead has histone acetylation activity. Ectopic over-expression of AtMBD9 and transient DNA demethylation promotes flowering and causes partial recovery of the normal branching phenotype. Co-immunoprecipitation assays suggest that AtMBD9 interacts in vivo with some regions of the FLC gene and binds to histone 4 (H4). Gene expression profile analysis revealed earlier up-regulation of some flower-specific transcriptional factors and alteration of potential hormonal and signal transducer axillary branching regulatory genes. In accordance with this result, AtMBD9 itself was found to be localized in the nucleus and expressed in the flower and axillary buds. Together, these results suggest that AtMBD9 controls flowering time and axillary branching by modulating gene expression through DNA methylation and histone acetylation, and reveal another component of the epigenetic mechanism controlling gene expression.

Keywords: AtMBD9, methylation, acetylation, epigenetics, Arabidopsis, histone.

INTRODUCTION

Epigenetic regulation in eukaryotic cells is performed by a complex array of signaling connections among small RNA species and also by chromatin remodeling. The latter occurs at two possible levels: DNA methylation and histone modification. DNA methylation occurs on more than 30% of cytosine residues (CpG) in some plants (Gruenbaum et al., 1981) and more than 60% in mammals (Gruenbaum et al., 1981; Razin et al., 1984). Specific amino acid residues of histones, usually lysines within the N-terminal tail, are often subjected to a number of covalent post-translational modifications, including acetylation, ADP-ribosylation, methylation, phosphorylation or ubiquitination (Wolffe and Hayes, 1999).

Over the last few decades, a number of lines of evidence have led to the suggestion that CpG methylation in eukaryotes is correlated with gene silencing. Recently, methylation of certain arginines in histones has been shown to regulate flowering time in Arabidopsis (Niu et al., 2007; Schmitz et al., 2008). However, specific histone modifications have different effects on gene expression. For example, acetylation of histone H3 and H4 (He et al., 2003; Peng et al., 2006) and methylation of histone H3 K4 (He et al., 2004) and H3 K36 (Zhao et al., 2005) are associated with activation of FLOWERING LOCUS C (FLC), a gene encoding a transcription factor that controls flowering time in Arabidopsis (Michaels and Amasino, 1999; Sheldon et al., 1999). In contrast, methylation of H3 K9 and H3 K27 (Bastow et al., 2004) is associated with FLC repression. In addition, there is a strong functional correlation between histone modifications and cytosine DNA methylation (Tariq and Paszkowski, 2004; Mathieu et al., 2007). Some chromatin modifiers such as KRYPTONITE, an H3K9-specific histone methyltransferase, regulate DNA methylation in order to enhance
5-methylcytosines binding at particular regions of DNA and subsequently allow interaction with specific histone residues within the chromatin (Jackson et al., 2002, 2004). The protein structure that binds to the methylated CpG dinucleotide is called the methyl-CpG binding domain (MBD).

Chromatin modifiers often form protein complexes with MBD proteins. Such a situation has been found in mammalian cells, in which MeCP1, a histone deacetylation complex, includes the MBD2 protein (Feng and Zhang, 2001). In Arabidopsis, the DECREASE IN DNA METHYLATION 1 (DDM1) protein co-localized with MBD2 (Feng and Zhang, 2001). Likewise, the AtMBD7 protein also interacts with arginine methyltransferase (PRMT11) (Scebbà et al., 2007). Twelve putative MBD genes (AtMBD1–AtMBD12) have been identified in the Arabidopsis genome (Berg et al., 2003; Springer and Kaeppler, 2005). Six MBD proteins (AtMBD1, 2, 4, 5, 6 and 7) showed specific binding capacity for the methylated CpG sequence in vitro. These proteins did not show DNA demethylase activity; however, AtMBD6 demonstrated histone deacetylation activity when a plant extract was treated with the recombinant protein (Zemach and Grafi, 2003).

Loss-of-function analysis of AtMBD9 mutant lines (atmbd9-1 to atmbd9-3) showed early flowering, increased axillary shoot branches, short plants (Peng et al., 2006), pale leaves and low-seed-yield phenotypes. Early flowering in these lines is due to the down-regulation of FLC, which is caused by reduced H3 and H4 acetylation levels at some regions of that locus. The mechanism by which AtMBD9 triggers a reduction in acetylation at FLC, and the molecular explanation for the other phenotypic defects, have not yet been determined.

In this study, we aimed to investigate the precise role of AtMBD9 in modulating the flowering and axillary branching phenotypes through biochemical functional characterization of AtMBD9 protein in vivo and in vitro. We found that AtMBD9 controls gene expression by modifying chromatin structure directly by acetylating histones and indirectly by decreasing the global methylation level of the DNA. Loss of function led to alteration of expression of a number of genes related to flowering and axillary branching pathways in Arabidopsis. AtMBD9 is an example of a transcription factor with two potential epigenetic mechanisms that influence multiple phenotypes.

RESULTS

Over-expression of AtMBD9 cDNA rescues the wild-type phenotype in the atmbd9 mutant

AtMBD9 was originally identified in a reverse-genetic screen in which the mutants showed early flowering and multiple axillary branch phenotypes (Peng et al., 2006). To further confirm the direct influence of AtMBD9 on these phenotypes, the AtMBD9 cDNA sequence was initially cloned under the control of the constitutive CaMV 35S promoter (P35S) in the pEGAD vector. After transformation into atmbd9 and wild-type lines, the transgenic plants were unhealthy and showed a high level of mortality after germination. As it was not possible to obtain a genetically stable homozygous AtMBD9 transformant line using this strategy, the 35S promoter was replaced by 2023 bp of the AtMBD9 native 5′ upstream regulatory sequence (P2000). Some transgenic lines obtained using this strategy were healthy and genetically stable for three generations when transformed into either atmbd9 or wild-type plants. The atmbd9 lines transformed with this construct flowered almost simultaneously with wild-type plants, and had a similar number of axillary branches. Wild-type Arabidopsis plants transformed with the same construct showed fewer axillary branches and earlier senescence than did untransformed plants, with some of these transgenic lines having only the bolting stem. These results support the view that expression levels of AtMBD9 affects these traits, with over-expression leading to the opposite phenotype to that which occurred in the mutant lines. The plants transformed with 35S–AtMBD9 showed a higher level of AtMBD9 expression than wild-type and those transformed with P2000–AtMBD9. However, the expression did not reach wild-type levels when atmbd9 was transformed with P2000–AtMBD9 (Figure 1). The early flowering phenotype correlated with a low expression level of FLC in the atmbd9 lines. Accordingly, transgenic P2000–AtMBD9 and 35S–AtMBD9 plants restored FLC expression to the wild-type level (Figure 1). In the fourth generation of either wild-type or atmbd9 plants transformed with the P2000–AtMBD9 construct, the phenotype of the homozygous lines started to segregate, and this was due to silencing in the transgene. This situation was noticed in four transgenic lines.

The DNA methylation level modulates the phenotype in the atmbd9 mutants

FLC expression is lower in the atmbd9 lines and is correlated with a lower acetylation level at the FLC locus than is present in wild-type plants. However, it is important also to analyze the in vivo DNA methylation pattern at this locus. Therefore, the DNA cytosine methylation within the 867 bp upstream of the start codon, in exon 1 and in only the 358 bp intron 1

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of the *FLC* gene was analyzed using the sodium bisulfite DNA sequencing technique. The results revealed the presence of 18 methylated cytosine sites within the *FLC* promoter, exon 1 and intron 1 sequences in the *atmbd9* lines. These methylated cytosine sites were not found in the Col wild-type counterparts (Figure 2a). Fifteen of these sites showed the CpHH motif (where H is A, C or T) and three the CpG motif. Methylation was verified by using PCR to amplify the genomic DNA after digestion with *Mcr*BC endonuclease, which cleaves DNA containing methylcytosine (Figure 2b), and also by using a methylation-sensitive restriction enzyme coupled with Southern blot analysis (Figure S1). To whether the increase in cytosine methylation level occurred globally across the genome, and also to investigate the direct influence of *AtMBD9* over-expression on the DNA methylation level, global DNA methylation analysis was carried out using an ELISA-like reaction and *atmbd9*, wild-type, P2000–AtMBD9/*atmbd9*-1 and P2000–AtMBD9/WT transgenic DNA. The *atmbd9* genomic DNA was found to have approximately 15% more methylation than the Col wild-type Arabidopsis plants (Figure 3). This result was verified by the use of methylation-sensitive restriction enzymes coupled with Southern blot analysis (Figure S2). In contrast, the percentage of methylated cytosine was similar to the wild-type level in the transgenic P2000–AtMBD9/*atmbd9*-1 line, and the P2000–AtMBD9/WT DNA had a slightly lower level of methylated cytosine residues (Figure 3). Therefore, AtMBD9 clearly helps regulate the global level of cytosine methylation.

To investigate the effect of cytosine methylation on flowering time and the axillary branching habit in *atmbd9* and Arabidopsis wild-type, DNA methylation levels were transiently reduced by treating the plants at an early stage with the cytosine methyl transferase inhibitor 5-azacytidine (5-azaC) (Jones, 1985; Haaf, 1995). After treating the seedlings with 0.5 mM 5-azaC for 5 days, growth of all plants slowed down by 1 week, and the differences in flowering time and axillary branching number between wild-type and *atmbd9* were significantly reduced (Table 1 and Figure 4a,b). In addition, there was partial recovery of *FLC* expression after treatment with 0.5 mM 5-azaC (Figure 4c) because of the partial reduction in DNA methylation (Figure 4d). Therefore, the global DNA methylation
CmpG sites
AtMBD9 acetylates histones but does not demethylate plants.

flowering time and shoot branching pattern in the level of the treated plants was reduced, and changed the flowering time and shoot branching pattern in the atmbd9 lines such that it was closer to that seen in wild-type plants.

AtMBD9 acetylates histones but does not demethylate C\textsuperscript{5m}G sites in vitro
Recombinant full-length AtMBD9 was expressed and purified from insect cells and used for various biochemical assays. In addition, specific polyclonal antibodies against AtMBD9 were raised in rabbits, and used in some of these assays (Figure S3). Loss of function of AtMBD9 leads to an increase in the DNA methylation level. In addition, this level was decreased in the atmbd9-complemented transgenic lines (Figure 3). In view of this result, a DNA demethylation assay based on methylation endonuclease sensitivity was performed using the recombinant AtMBD9 protein. Unexpectedly, the recombinant AtMBD9 did not show any detectable DNA demethylation activity when an in vitro-methylated FLC DNA sequence was used as the substrate (Figure S4), but did show significant acetylation activity in vitro. The acetylation activity was measured at various time points using various amounts of recombinant protein. The increase in activity was found to be directly correlated to the amount of recombinant protein used in the reaction (Figure 5a). The acetylation activity was confirmed using the gel-based method described by Brownell et al. (1999) (Figure 5b).

The acetylation levels of histones H3 and H4 in the atmbd9 and wild-type were measured in vivo using an immunoblotting technique. In agreement with the in vitro enzymatic assay, histone H3 and H4 extracted from the wild-type have a higher acetylation level than those extracted from the atmbd9 mutant. The acetylation level was even higher in the over-expressed P2000–AtMBD/WT lines (Figure 5c). In addition, histone H4 was detected in the AtMBD9 immunoprecipitated complex when it was probed using acetylation-specific H4 antibodies in the Western blot (Figure 5d,e). Histone H3 was not detected in the same complex, which may reflect specificity and a direct functional interaction between H4 and AtMBD9.

ChIP assay reveals a direct interaction between AtMBD9 and FLC
It has been shown that the FLC expression level is regulated by various transcription factors and also by environmental conditions such as light and temperature (Mouradov et al., 2002). Peng et al. (2006) previously reported that loss of AtMBD9 function inhibits FLC expression by reducing the acetylation level in some regions within this locus. By using AtMBD9-specific antibodies in a chromatin immunoprecipitation (ChIP) assay, we found that AtMBD9 binds to three regions of FLC (B, D and I) previously defined by Bastow et al. (2004) and Peng et al. (2006) (Figure 6). Regions B and D span part of the promoter and the first exon, and part of the first intron of FLC, respectively, and region I covers the junction region of intron 6 and exon 7. Compared with the other regions, the highest degree of interaction was found in region I (Figure 6f).

Sodium bisulfite sequence analysis of FLC region B in the atmbd9 mutant revealed the presence of two methylated CpG sites and several CHH sites (where H is A, C or T) within this region (Figure 2). These methylated cytosines probably represent AtMBD9 binding sites in wild-type chromatin. The binding may be followed by demethylation of these cytosines by another protein, which does not occur in the absence of AtMBD9.

AtMBD9 is localized in the nucleus
AtMBD9 contains two nuclear localization signals (NLS) that reside within the N-terminal and centric domains of the protein. The PKRKKTS and KLVRKKK amino acid NLS motifs start at the 335 and 1348 amino acids of AtMBD9, respectively (Figure S3). Full-length AtMBD9 fused with GFP showed cytoplasmic aggregations and did not localize to a specific cellular compartment when transiently expressed in either Arabidopsis cells, B21 tobacco suspension cells or onion epidermal cells. This might be due to the large size of the GFP–AtMBD9 fusion protein. Therefore, the parts of the AtMBD9 that include the NLSs were separately fused with GFP and used for the locali-
The results showed that the peptides containing the N-terminal and the centric NLSs are both able to direct the GFP to the nucleus of onion epidermal cells (Figure 7). This result provides direct evidence that AtMBD9 is a nuclear protein and contains two functional NLSs.

AtMBD9 is expressed in the shoot apical region and shoot branching sites

As AtMBD9 regulates FLC transcription and shoot branching in Arabidopsis, it is informative to investigate the spatial and temporal expression pattern of AtMBD9, and compare the
resulting information with the expression pattern of FLC and the occurrence of shoot branching. Using RT-PCR, Berg et al. (2003) demonstrated that AtMBD9 is expressed in the rosette leaves, flowers and stems, but no AtMBD9 transcript was detected in the root, green siliques and seeds. To examine AtMBD9 expression in more detail throughout Arabidopsis development, we fused the AtMBD9 promoter (PAtMBD9) with the GUS reporter gene, and transformed this construct into Arabidopsis plants. Expression of the GUS gene under the control of the AtMBD9 promoter was detected at various developmental stages by histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc).

At 7 days after germination, AtMBD9 expression was detected in the vascular system of the cotyledons and hypocotyls, but not in root tissue. The strongest GUS staining was found in the shoot apical region (Figure 8a) where dividing cells reside and FLC shows the highest expression (Michaels and Amasino, 2001). After further growth of the Arabidopsis plants, AtMBD9 was expressed in the vascular tissues of rosette leaves, old inflorescence stems and cauline leaves (Figure 8b–d). High expression of AtMBD9 was always detected in rosette regions where secondary rosette branches are produced (Figure 8b), and at inflorescence junctions where the outgrowth of tertiary branches occurs (Figure 8c). This pattern fits well with the fact that AtMBD9 regulates the Arabidopsis shoot branching trait. In addition, 14-day-old plants showed strong PAtMBD9–GUS activity in root junction parts where lateral roots are formed (Figure 8b). In flowers, PAtMBD9–GUS staining was visible only in the vascular tissues of sepal, the filament and the receptacles (Figure 8e). In green siliques, AtMBD9 was expressed only at the junctions between siliques and pedicels (Figure 8f).

Transcriptome profiling of atmbd9-1 revealed the molecular events associated with the phenotypic variation

There is an increase in overall DNA methylation in the atmbd9 mutant, and one would expect this to have a significant effect on the transcriptome. Therefore, a set of whole-genome microarray experiments were performed on apical bud tissue isolated from the first-producing rosette leaves and buds of atmbd9-1 and wild-type plants. Bud tissue was chosen in the expectation that this would help explain differences in the axillary branching phenotype. The microarray results showed that there are 333 genes whose expression is significantly different in the atmbd9 mutant, with 158 and 175 genes up- and down-regulated, respectively. These genes were classified based on their biological functions in the plant (Table S1 and Figure S5). The expression level of 21 representative genes was confirmed using RT-PCR (Figure S6). Proportionately there are more up-regulated genes encode proteins that are localized in the nucleus and associated with transcription factor activity or DNA/RNA binding and involved in developmental processes compared to those that are down-regulated.

The expression profile contains genes with both direct and indirect relationships to flowering, branching and hormonal metabolic pathways. For example, transcriptional factors associated with flower initiation and developmental pathways, such as SEPALLATA 1 and 3 (SEP1 and SEP3) and SQUAMOSA promoter binding protein-like 3 and 4 (SPL3 and SPL4) were prematurely expressed at a high level in the atmbd9 mutant (Table S1), indicating their involvement in the early flowering phenotype in atmbd9-1. It is well-demonstrated that hormonal balance plays an important role in flowering and branching in plants. Therefore, it is not surprising that hormone-related genes such as ETHYLENE-INSENSITIVE3, EIN3/EBF2-BINDING F BOX PROTEIN 2
At5g25350), ETHYLENE RECEPTOR SUBFAMILY 1 (ERS1), At2g40940, GA REQUIRING 4 (GA4), the GA-related gene S-ADENOSYLMETHIONINE-DEPENDENT METHYLTRANSFERASE/GIBBERELLIN CARBOXYL-O-METHYLTRANSFERASE (GAMT2) and the gene encoding cytochrome P450 (CYP79B2, At4g39950) were differentially expressed in the atmdb9 mutant (Table S1). This latter gene encodes a protein that converts tryptophan to indo-3-acetaldoxime.

Figure 6. Chromatin immunoprecipitation assays (ChIP) in wild-type and atmdb9-1 Arabidopsis.
(a) The genomic structure of FLC and the regions tested in the ChIP assay. Coding regions are indicated by black boxes.
(b) PCR products of input DNA from the wild-type used in the ChIP assay.
(c) Control ChIP assay using chromatin extracted from wild-type plants and without using AtMBD9 antibodies.
(d) Control ChIP assay using chromatin extracted from atmdb9-1 and AtMBD9 antibodies.
(e) ChIP assay performed using chromatin extracted from wild-type Arabidopsis and the AtMBD9 antibodies.
(f) Signal intensities were normalized relative to RNA polymerase 2 ChIP control reactions (RNA-P), and the mean fold changes (±SD) of three experiments are shown. Actin-2/7 was used as a PCR-negative control for the ChIP assays.

Figure 7. Subcellular localization of AtMBD9.
(a) GFP fused with NLS expressed in the nucleus of onion epidermal cells.
(b) Nucleus of onion epidermal cells stained with 4’-6-Diamidino-2-phenylindole (DAPI).
(c) Merged image of (a) and (b).

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glucosinolate biosynthesis (Hull IAOx), a precursor that is required for IAA and indole glucosinolate biosynthesis (Hull et al., 2000).

In addition, genes involved in signal transduction pathways that lead to multiple axillary branches were differentially expressed in the atmbd9 mutant. These include the plasma membrane intrinsic protein subfamily PIP2 (At1g37170) and the leucine-rich kinase protein (At5g25930), which are involved in the inositol phosphate metabolism pathway, and SETH2 (At3g45100) (Gillmor et al., 2005), which is involved in the first step of the glycosylphosphatidylinositol-anchored biosynthesis pathway.

The genomic DNA of atmbd9 is hypermethylated. Coincidentally, atmbd9-1 showed expression alteration of some genes encoding putative nucleic acid methyl transferases and binding proteins, including up-regulation of methyltransferase type II (At1g01660), the jmjC domain-containing protein At3g20810, and ribosomal RNA RmJ/FtsJ-like methyltransferase (At5g13830), and down-regulation of the methylcytosine-binding protein, VARIANT IN METHYLATION 4 (VIM4) (At1g66040) (Woo et al., 2007).

**DISCUSSION**

**AtMBD9 expression is critical for normal growth and development**

AtMBD9 controls gene expression directly through histone acetylation and indirectly through DNA methylation. It was previously shown that mutants in this gene had altered developmental patterns, particularly with regard to flowering time and shoot branching (Peng et al., 2006). In the present study, over-expression of AtMBD-9 under the control of the CaMV 3SS promoter generally caused plant death at an early stage, and those plants that did survive were genetically unstable. When expression was regulated by the native promoter, the resulting construct was able to rescue most of the phenotypes of the mutant gene. However, these plants eventually showed genetic instability as well. Therefore, tight control of AtMBD9 gene expression is important for normal growth and development.

When the AtMBD9 promoter sequence was fused to the GUS reporter gene, AtMBD9 was found to be highly expressed in the shoot apical region and the transition zone where transition from shoot meristem to floral meristem occurs (Figure 8a). High expression of AtMBD9 was also detected in the regions where lateral shoot branches (inflorescences) are produced (Figure 8b,c). The phenotypic alteration associated with AtMBD9 mutations, and the expression pattern seen in these experiments during growth and development, reflect the biological function of this protein in flowering and axillary branching in Arabidopsis.

**Increased DNA methylation is associated with the atmbd9 mutant phenotypes**

The molecular events associated with the loss of function of AtMBD9 in Arabidopsis provide an important indication of its role in the regulation of gene expression. In these mutants, there is lower expression of the flower repressor gene FLC due to low histone acetylation (Peng et al., 2006) and a high DNA methylation level (Figure 2) at this locus. The normal time of flowering was restored in atmbd9 mutants either after treatment with 5-azaC or complementary genetic transformation. These both led to a corresponding increase in FLC expression. In addition, both 5-azaC and complementation of the atmbd9 mutation led to partial recovery of the wild-type branching pattern, although the target genes involved in this process are still not known.

Mutations within DECREASE IN DNA METHYLATION1 and 2 (DDM1 and DDM2), DNA METHYLTRANSFERASE1 (MET1) (Vongs et al., 1993; Kakutani et al., 1996), and the DNA demethylase gene, REPRESSOR OF SILENCING GENES1 (ROS1) (Agius et al., 2006) altered the overall level of cytosine methylation and caused various developmental defects in Arabidopsis. This effect on DNA methylation level led to different effects on flowering time depending on the Arabidopsis ecotypes studied. DNA hypomethylation in ddm1 and met1-1 mutations caused late flowering in Columbia and Landsberg erecta (Kakutani, 1997; Kankel et al., 2003), but hypomethylation induced by vernalization or 5-azaC treatment promoted flowering in the vernalization-responsive Arabidopsis ecotype C24 (Burn et al., 1993; Finnegan et al., 1998). However, in both cases, the FLC expression level was critical for determining flowering time.
but these studies did not indicate whether the DNA methylation status of the FLC had been changed. However, in our study, we found that down-regulation of FLC in the early flowering atmbd9 mutant was directly due to methylation in the DNA sequence (Figure 2) and reduction of the acetylation levels (Peng et al., 2006).

As inactivation of AtMBD9 causes DNA hypermethylation in Arabidopsis, DNA demethylation activity was one of the putative functions of AtMBD9 that we investigated in this study. However, the full-length recombinant AtMBD9 protein produced in insect cells did not show any DNA demethylase activity when assayed in vitro. Although the possibility that multiple protein complexes are required for the AtMBD9 DNA demethylation activity cannot be ruled out, based on the in vitro assay, the simplest explanation is that the methylation status of the genomic DNA is influenced indirectly by AtMBD9. Transcription profile analysis of atmbd9-1 revealed expression alterations in four nucleic acid methyltransferase genes (METHYLTRANSFERASE type 11, RRMJ/FTSJ-LIKE METHYLTRANSFERASE, jmjC domain-containing protein, and VIM4) (Table S1). These changes in gene expression may have an impact on the DNA methylation status in the atmbd9 mutant lines.

AtMBD9 modulates gene expression directly through histone acetylation

In order to determine the specific biochemical function of AtMBD9 in controlling gene expression, the recombinant protein was tested for histone acetylation, deacetylation and some potential histone methyltransferase activities. Enzymatic activity assays showed that full-length recombinant AtMBD9 catalyzes the acetylation reaction in vitro (Figure 5a,b). AtMBD9 does not code for a well-defined histone acetyl transferase catalytic domain. Thus, there must be a novel histone acetyl transferase domain within AtMBD9 given the in vitro acetylation activity and the presence of a BRM0/Bah domain as is usually found in other histone acetylation proteins (Dhalluin et al., 1999; Goodwin and Nicolas, 2001; Hassan et al., 2006).

In agreement with the enzymatic function in vitro, mutant lines lacking AtMBD9 activity showed significantly lower overall histone H3 and H4 acetylation levels than did wild-type Arabidopsis (Figure 5c). Results obtained from immunoprecipitation of the total histones suggest that AtMBD9 also binds to acetylated H4 histones (Figure 5d). The reduction in acetylation level in the atmbd9 lines was previously observed by Peng et al. (2006), who immunoprecipitated FLC chromatin using acetylated histone H3 and H4 specific antibodies in a ChIP assay.

AtMBD9 binds in vivo to several FLC chromatin regions covering part of the promoter and transcription initiation site (region B), the first exon (region D) and a sequence within intron 6 and exon 7 (region I) (Figure 6). It has been reported that chromatin in these regions is subjected to several histone alterations leading to regulation of FLC expression. For example, the FLC region B overlaps with a low acetylated H3 region upstream of the transcriptional start site in Arabidopsis after vernalization (Sung and Amasino, 2004). In addition, mutations in the flowering time locus of the autonomous pathway (FVE) led to an increase in acetylation in region D of FLC in earlier-flowering plants (Ausin et al., 2004). The AtMBD9 ChIP library was highly enriched with the FLC sequence that covers the junction between intron 6 and exon 7 (region I). This region was found to interact with FLOWERING LOCUS D (FLD), LYSINE-SPECIFIC DEMETHYLASE 1 and the RNA-binding protein FCA that together mediate H3K4 demethylation in FLC (Liu et al., 2007). Together, these results confirm that AtMBD9 binds to DNA and histones, and therefore can modulate key regulatory regions across the FLC chromatin by increasing their acetylation level and consequently enhancing FLC gene expression. The chromatin regions B and D of FLC that bind to AtMBD9 closely flank region C, which has a low histone H4 acetylation level in FLC of atmbd9 mutants, as previously determined by Peng et al. (2006). The variations between the two ChIP assays could be due to a difference between the AtMBD9 DNA binding and histone acetylation active sites on the compact nucleosomal structures, or due to different interaction and detection capacities between AtMBD9 and the acetylated histone H3 and H4 antibodies used in the assays.

AtMBD9 controls flowering time and lateral branch formation through a novel epigenetic mechanism

AtMBD9 encodes a protein containing a number of domains that are predicted to have activities involved in the epigenetic control of gene expression through chromatin modifications such as histone acetylation and methylation, and the 5-methylcytosine (m5C) binding domain that allows binding to DNA. It has been previously reported that methylated DNA and histone modification have integral and overlapping roles in epigenetic regulation in plants (Tariq and Paszkowski, 2004; Vaillant and Paszkowski, 2007; Saze et al., 2008; Zhang et al., 2008) as well as in mammals (Allen and Antoniou, 2007; Sridhar et al., 2007; Vincent et al., 2007). AtMBD9 affects both on the DNA methylation and in vitro histone acetylation activity, and provides a direct link between DNA methylation and histone alterations. These features functionally distinguish AtMBD9 from other MBD proteins.

The mechanism by which AtMBD9 interacts with chromatin is not completely understood. However, based on our findings, we propose the following model for its action (Figure 9): (i) AtMBD9 binds to chromatin via methylated DNA and then to histone H4, (ii) this binding leads to acetylation of the histones, and (iii) this leads to demethylation of DNA by another protein. A similar mechanistic link between histone acetylation and DNA demethylation has
been previously suggested to control gene expression in human embryonic kidney (HEK) cells. In this case, DNA demethylation was followed by histone acetylation (D’Alessio et al., 2007). Another pathway was hypothesized to describe the silencing of some tumor suppressor genes in humans (Belinsky et al. 2003; Issa, 2004; Brock et al., 2007), in which the MBD protein binds to abnormally methylated CpG and serves as a ligand for the histone deacetylation protein. However, AtMBD9 can be recruited to the CpG sites as well as acetylating histones. In addition to this pathway, it is possible that AtMBD9 enhances DNA methylation by up-regulating some DNA methylation-related genes. The absence of functional AtMBD9 in a cell would thus lead to accumulation of methylated cytosines in the genome and consequently to a significant change in global gene expression.

In conclusion, the atmbd9 mutation causes changes in flowering time and axillary branching. The AtMBD9 protein has a variety of domains that interact with chromatin, and in this study was found to acetylate histones in vitro. In addition, an increase in the global DNA methylation and a reduction in histone acetylation levels were observed in the mutant line, while the lines over-expressing this protein showed the opposite effect. As an example, the atmbd9 line shows low acetylation in the chromatin regions of the FLC gene, and an increase in DNA methylation at some cytosine residues. This explains the changes seen in FLC expression. This information supports the hypothesis that there is a cooperative mechanism between DNA methylation and histone modifications that controls gene expression.

**EXPERIMENTAL PROCEDURES**

**Plant material, growth conditions and treatment with 5-azaC**

For 5-azaC treatments, seeds were surface-sterilized, cold treated for 2 days at 4°C, and germinated for 8 days under the same growth conditions on 1 mm Whatman filter paper saturated with either water or a solution of 0.3, 0.5 or 1 mM 5-azaC (Sigma-Aldrich, http://www.sigmaaldrich.com/). The solutions were prepared freshly every day. After germination, seeds were grown on regular LA4 soil (Sunshine, http://www.sungro.com/index.php) under normal conditions.

**Cloning of the AtMBD9 cDNA and plant genetic transformation**

Total RNA was extracted from wild-type plant leaves using an RNasy plant mini kit (Qiagen, http://www.qiagen.com/), and the cDNA was amplified using primer pair F-MBD9 (5’-CTGACCGG-TATGGAACCCACTGATTCTAAGG-3’) and R-MBD9 (5’-CTAGG-ATCCCTCGGGTTCTTTCCTTTG-3’). The amplified fragment was digested with AgeI and Smal, and cloned into the binary pEGAD expression vector after removing the EGFP coding region from the plasmid (Cutler et al., 2000). The resultant construct was named P3SS-AtMBD9-pEGAD. Subsequently, the 3SS promoter of the P3SS-AtMBD9-pEGAD was replaced by putative promoter residues within the 2023 bp upstream of the AtMBD9 coding region. The putative promoter was amplified from the genomic DNA by PCR using primer pair F-Promoter (5’-CCACCCACCGTGATCTAACGTTACT-3’) and R-Promoter (5’-GTCTACCTGGGGTATCTGGCAGGTATGAGGTGTT-3’), and cloned into the binary pEGAD vector. The resultant construct was named P2000-AtMBD9-pEGAD. In order to fuse the AtMBD9 promoter to the GUS reporter gene, a DNA fragment (1 kb) upstream of the AtMBD9 coding region was amplified from the Arabidopsis genome by PCR using primer pair F-GUSMBD9 (5’-ATCTTCTAGAGTCGTC-3’ and R-MBD9 (5’-ATCTTCTAGAGTCGTC-3’)-ATCTTCTAGAGTCGTC-3’). The PCR product was digested with XbaI and Ncol, and cloned between the XbaI and Ncol sites of the GUS reporter gene fusion binary vector pCAMBIA3301 (Cambia Institute, http://www.cambia.org). All constructs were amplified in Escherichia coli DH10b cells and transformed into Agrobacterium tumefaciens EHA105 cells. Wild-type plants and atmbd9 mutant lines were transformed with P3SS-AtMBD9-pEGAD and P2000-AtMBD9-pEGAD using the floral dipping method. Transgenic plants were selected by spraying with BASTA (Glufosinate) herbicide (Bayer Crop Science, http://www.bayercropscience.ca).

For determination of subcellular localization, the AtMBD9 DNA sequences coding for the N-terminal NLS (562–1587 bp) and the centric NLS (3442–4620 bp) were independently cloned in-frame with the GFP protein under the control of the 3SS promoter. The N-terminal NLS cDNA sequence was amplified by PCR using primer pair F-NLSS (5’-CGCGGATCTGATCTAACGTTACTAAGC-3’) and
Expression and purification of full-length AtMBD9 and in vitro activity assay

The full-length AtMBD9 recombinant protein was expressed and purified using a baculovirus IMPACT system (New England Biolabs). The AtMBD9 cDNA was amplified by PCR using primer pair F-BacMBD9 (5'-GCTCTAGAATGGAACCCACTGATTCTACTAACTAGA- GC-3') and R-BacMBD9 (5'-TTCTTTTTCCGCGCGATCC- TCGGGT-3'). After digestion with BamHI and XbaI, the PCR products were cloned into the pRlLT2-GFP plasmid (kindly provided by Dr Robert Mollen at Guelph University) and transformed by particle bombardment into onion epidermal cells.

Peptide antibodies

The DNA sequence (4350–5235 bp) coding for a partial AtMBD9 peptide was amplified by PCR using primer pair F-ProMBD9 (5'-CGATTTCCATATGGAAACCGAATTCTGAAGGAA-3') and R-ProMBD9 (5'-CGGGAATTCAGCAATAAATCTGTACCGTTGTTT-3'). The PCR product and plasmid were digested with Sall and Smal cloned into the pTY2 vector (New England Biolabs, http://www.neb.com/nebecomm/default.asp). The construct was amplified in E. coli DH10B cells and transformed into E. coli expression host strain ER2566 cells. The protein was expressed and purified using the IMPACT system (in vitro mediated purification with an affinity chitin-binding tag) (New England Biolabs), according to the manufacturer’s instructions. The AtMBD9 peptide was used as an antigen to produce polyclonal antibodies in rabbits using the service obtained from Cedarlane (http://www.cedarlanelabs.com), and the antibodies were purified using the method described by Smith and Fisher (1984).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described previously by Johnson and Bresnick (2002). Purified AtMBD9 antibodies were used in the assay to precipitate AtMBD9-associated DNA in the chromatin of wild-type and atmbd9 leaves. The resultant DNA was used to amplify FLC regions using the primer pairs given by Bastow et al. (2004) and Patnaik et al. (2004). The amounts of PCR products were quantified using IMAGEJ software (Abramoff et al., 2004).

Protein immunoprecipitation and immunoblotting

Total proteins were extracted from leaves of Arabidopsis seedling lines grown on MS medium for 10 days. The leaves were harvested, ground in liquid nitrogen and extracted using 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM PMSF, 1 mM EDTA, 0.1% v/v Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and complete protease inhibitor cocktail tablets (Roche). Histones were separated on 12.5% a polyvinylidene difluoride (PVDF) membrane (Roche) for 3 h at 120 volts. Histones were digested with McrDII and DamI and were identified using the pVIC108 vector (New England Biolabs) at the Spodoptera frugiperda-21 (Sf21) insect cells using the BacVector-3000 DNA kit (Novagen, http://splash.emdbiosciences.com). Protein purification was performed according to the protocols described by Pradhan et al. (1997) and Patnaik et al. (2004). Recombinant AtMBD9 was dialyzed twice overnight at 4°C in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM PMSF, 1 mM DTT, complete protease inhibitor cocktail tablets (Roche) and 50% v/v glycerol. The histone acetylation activity (HAT) of the recombinant protein was measured using a HAT activity colorimetric assay kit (Biovision, http://www.biovision.com).

DNA methylation analysis

The DNA global 5-methylcytosine content in the wild-type and atmbd9 and transgenic lines was measured using a Methyлamp global DNA methylation quantification kit (Epigentek, http://www.epigentek.com). Standard curves of the amount of the methylated DNA were generated using the kit’s positive and negative controls. Methylated cytosines within FLC were identified using sodium bisulfite sequencing methodology. Genomic DNA for the various genotypes was treated with a bisulfite kit (Qiagen). Subsequently, the FLC sequences were amplified by PCR using the treated DNA as template and the degenerated primer pairs listed in Table S2. The PCR products were cloned into pGEM-T Easy vector (Promega, http://www.promega.com/), and at least 20 clones of each construct were sent for sequencing at the University of Guelph sequencing facilities. As a control reaction, the FLC DNA fragment was amplified from the genomic DNA by PCR, cloned into the pGEM T-easy vector, propagated in E. coli JM110 (dam and dcm) (Stratagene, http://www.stratagene.com/) and used in the sodium bisulfite reaction. For digestion with MspI (New England Biolabs), DNA was treated according to the manufacturer’s instructions, purified and used as a template in the PCR using FL-TF and FL-T3 primers (Table S2).

Microarray hybridization and analysis

Gene expression analysis was carried out using Operon Arabidopsis genome oligo set version 3.0 (Operon, http://www.operon.com/) long-oligonucleotide microarrays purchased from the laboratory of Dr David Galbraith (Department of Plant Sciences, University of Arizona, http://www.ag.arizona.edu/microarray/). Microarray hybridization was performed as described by Bueso et al. (2007). The microarray expression data were normalized and statistically analyzed using GENE.Spring GX 7.3.1 software (Agilent Technologies, http://www.home.agilent.com). The alteration in expression level of representative genes was verified by RT-PCR using primers shown in Table S3.

Quantitative RT-PCR

Total RNA was extracted from leaves as described above, and cDNA was synthesized using the AMV first-strand cDNA synthesis kit (Promega). Expression of FLC was detected by quantitative RT-PCR.
using the method and primer pairs described by Peng et al. (2006). To analyze AtMBD9 transcription by quantitative RT-PCR, the primers 5'-TGGAGGAAGCTAAGATGATGT-3' (forward) and 5'-CATGCGCAGGAGACTTATC-3' (reverse) were used.

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SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article:
Figure S1. FLC of the atmb9 mutant lines have more DNA methylation than the wild-type.
Figure S2. atmb9 mutant lines have more global DNA methylation than the wild-type.
Figure S3. Putative protein structure of AtMBD9 and the strategy used to produce the recombinant proteins in vitro.
Figure S4. DNA demethylation assay using the recombinant full-length AtMBD9 and the methylated CpG FLC DNA sequence amplified by PCR.
Figure S5. Functional categorization of the 332 genes differentially expressed in the atmb9-1.
Figure S6. Relative expression level obtained using the RT-PCR of some representative genes differentially expressed in the atmbd9-1 line.
Table S1. Genes that were differentially expressed in the atmbd9-1 axillary buds.
Table S2. Primers used to amplify the FLC locus from sodium bisulfite-treated DNA.
Table S3. Primers used in RT-PCR. Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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