# Methylated DNA-Binding Proteins from Arabidopsis[1]

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The 5-methylcytosines ($m^5C$) play a critical role in epigenetic control, often being recognized by proteins containing a methyl-CpG-binding domain (MBD). Database screening has identified at least 12 putative methyl-CpG-binding proteins from Arabidopsis; we have isolated corresponding cDNAs for seven of them. Despite variation in size and amino acid sequence, all seven proteins exclusively migrate into the nucleus as revealed by green fluorescent protein fusion protein assay, suggesting a relationship with chromatin structure. However, DNA-binding assays using bacterially expressed proteins and synthetic oligonucleotides containing $m^5C$ in CpGs showed only one to specifically bind, designated AtMBD5. Further analysis showed that AtMBD5 efficiently binds to $m^5C$ in CpNpN (N is A, T, or C) but not in CpNpG sequences, both frequently found in plant DNA. The other six proteins showed either nonspecific DNA binding or no ability to recognize $m^5C$. RNA-blot hybridization and immunoblot analysis indicated AtMBD5 to be present essentially in all tissues. Using green fluorescent protein driven by the authentic promoter, AtMBD5 was found to be actively expressed in pistils and root tips. Because $m^5Cs$ in CpG and CpNpN are considered to function in gene expression and gene silencing, respectively, the present results suggest that AtMBD5 may have distinct functions in regulation and/or self defense of genes in actively proliferating cells.

The DNA of higher eukaryotes contains 5-methylcytosine ($m^5C$) as up to 30% of total cytosine residues. In plants, methylation has been shown to predominantly occur in CpG and CpNpG sites (N stands for A, T, or C), but one study revealed that cytosines in CpNpN are also methylated (Oakeley and Jost, 1996). The methylation of cytosine is a post-replicative process catalyzed by DNA methyltransferases. To date, three types of these enzymes have been identified in plants; MET1 for maintenance of CpG methylation (Finnegan and Kovac, 2000), chromomethylase for de novo methylation of CpNpG (Bartee et al., 2001; Lindroth et al., 2001), and domain-rearranged methyltransfease, also for de novo methylation (Cao et al., 2000).

The physiological function of DNA methylation has been proposed to essentially be silencing of genes for which expression is undesired in given tissues (Yoder et al., 1997). Intensive studies on animal cells have revealed distinct examples, including X chromosome inactivation, imprinting of genes, and silencing of parasitic DNA such as retroelements and transposons (Robertson and Wolffe, 2000). In plants, mobilization of transposons is also reported to be repressed by DNA methylation (Miura et al., 2001). The molecular mechanism by which DNA methylation prevents transcription is considered to be mediated through changes of DNA structure caused by $m^5C$. Two features are proposed. One is direct repression due to methylated promoter regions blocking binding of transcriptional machinery, and the other is indirect repression due to alteration in the nucleosome structure affecting the whole chromatin conformation (Bird and Wolffe, 1999). Recent studies have indicated the latter to occur frequently and to play an essential role in controlling global gene expression (Bird, 2002).

Among proteins that complex with DNA to form chromatin, examples possessing a methyl-CpG-binding domain (MBD) may play a critical role in transcription repression (Bird, 2002). In animal cells, five MBDs have been identified so far, among which MeCP2 is best characterized (Bird and Wolffe, 1999). It is a single polypeptide of 486 amino acids, containing both an MBD and a transcriptional repressor domain, which efficiently binds to symmetrical $m^5C$ in CpG dinucleotides (Wakefield et al., 1999). Analyses have indicated that MeCP2 interacts with histone deacetylase, which globally represses gene expression (Jones et al., 1998; Nan et al., 1998). Protein-protein interactions also have been found between MBD2 and MBD3 (Hendrich et al., 2001). In this case, MBD2, which binds to methylated DNA, recruits MBD3, which lacks DNA-binding ability by itself but forms a corepressor complex with histone deacetylase and, thus, plays a critical role in mouse development. As a consequence, MBDs are considered to be key components in DNA methylation-mediated transcriptional silencing (Robertson and Wolffe, 2000).

Despite the importance in chromatin assembly and gene expression, studies on plant MBDs have been

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limited so far. Here, we report isolation and characterization of seven putative MBDs from Arabidopsis, among which only one was found to be able to specifically bind to $m^5Cs$ in CpG and CpNpN.

## RESULTS

### Isolation of cDNAs Encoding Putative AtMBD Proteins

When we initiated the present study, eight Arabidopsis genes encoding putative proteins possessing an MBD were found in the plant chromatin database (http://www.chromdb.org/index.html) and designated AtMBD (Arabidopsis MBD) with serial numbers 1 through 8 (Fig. 1A). The number of genes, however, subsequently increased, and, currently, 25 putative genes with MBDs are present in the database. In this study, however, we focused on the initially identified eight genes. The nomenclature was followed after the database except for AtMBD7A and AtMBD8A because different sequences were recently assigned to AtMBD7 and AtMBD8 (Zemach and Grafi, 2003; see "Discussion").

The potential MBDs in the different proteins generally have a low level of similarity, although some showed more than 50% similarity, such as AtMBD4 and AtMBD5 with AtMBD1 and AtMBD6, respectively (Fig. 1, B and C). All the AtMBDs are characterized by a lack of the transcriptional repressor domain, which MeCP2 and MBD1 of mammals typically possess. To determine whether or not these genes are transcribed in planta, a cDNA library derived from the whole plant of Arabidopsis was screened to identify the corresponding transcripts by PCR, using specific primers designed according to the predicted open reading frames. As a result, all cDNAs except for that of AtMBD3 could be isolated. Although some of the open reading frame in the database was based on prediction, the determined nucleotide sequences of these clones were identical with those predicted, indicating correct placement of

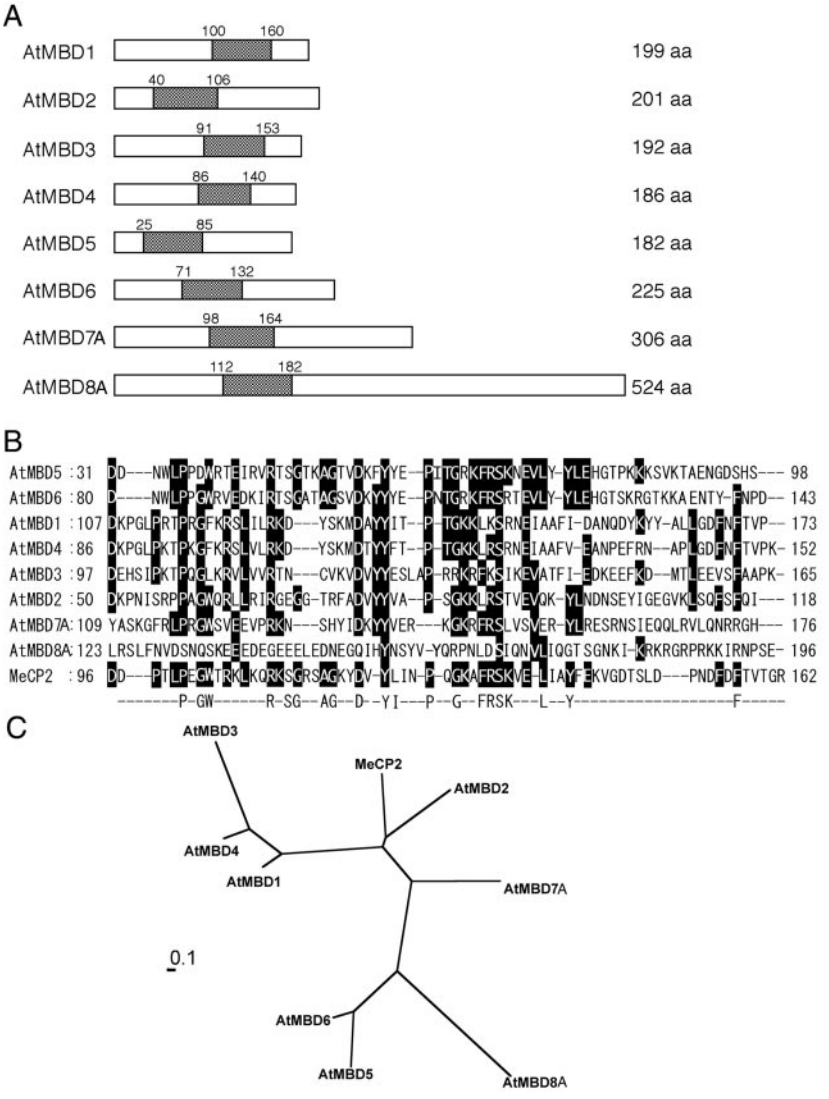


**Figure 1.** Comparison of eight AtMBD proteins. A, Schematic illustration of AtMBD protein structures. The size of each protein is indicated in amino acid numbers (aa), and each MBD region is indicated by a shaded box. B, Amino acid alignment of MBD regions. Amino acid residues identical in more than four proteins are indicated by shading. MeCP2 is of human origin. Gaps are introduced to best match the amino acids. The consensus amino acids among mammalian MBDs are shown on the bottom line. C, Unrooted phylogenetic tree. The relationship was calculated from whole sequences for the AtMBDs. The branch lengths represent numbers of substituted residues per site.

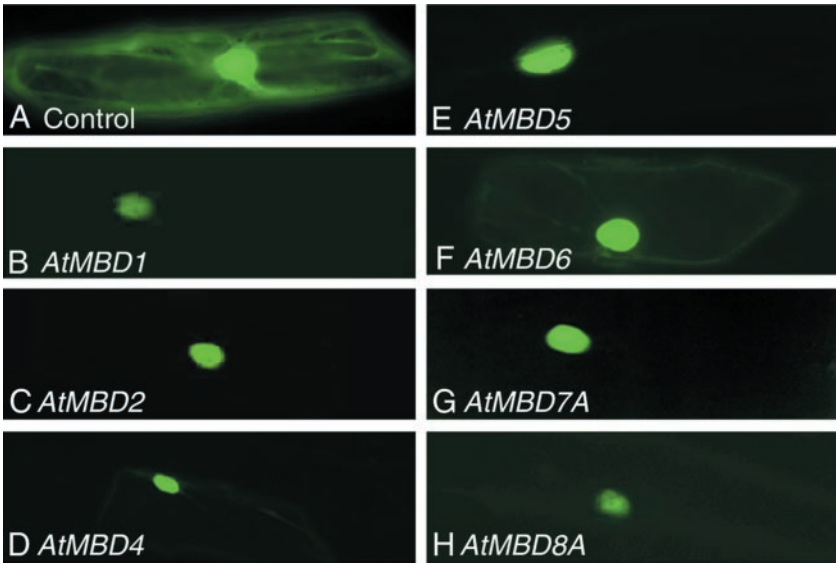


**Figure 2.** Nuclear localization of AtMBD-GFP fusion proteins. Onion bulbs were bombarded with gold particles coated with psGFP (A) or pAtMBD1-GFP (B) through to pAtMBD8A-GFP (H). Fused proteins were transiently expressed and individual cells were observed by epifluorescence.

splicing sites. However, two PCR products with different sizes were amplified for AtMBD7A. Because this amplification was observed in RT-PCR, it could due to alternative splicing of the AtMBD7A gene (data not shown). Because the larger cDNA encoded a small protein, truncated at the domain for methyl-DNA binding, it was eliminated from further analyses.

### Cellular Localization of AtMBD Proteins

Cellular localization of AtMBD proteins was examined with green fluorescent protein (GFP)-fused constructs. Plasmids containing cauliflower mosaic virus (CaMV) 35S promoter *AtMBD*::*GFP* genes were constructed and biolistically bombarded into onion (*Allium cepa*) epidermal cells. Epifluorescence analyses indicated that, whereas the control construct containing only *GFP* showed signals in cytoplasmic space and in nuclei, all *AtMBD*::*GFP* constructs demonstrated fluorescence exclusively in nuclei (Fig. 2).

### Binding Assay

To determine whether or not AtMBD proteins recognize and bind $m^5C$, glutathione *S*-transferase (GST)-AtMBD recombinant proteins were expressed in *Escherichia coli* and partially purified in glutathione-Sepharose columns. Each AtMBD protein was subjected to binding assay with double-stranded synthetic oligonucleotides of 50 bp containing five symmetrical $m^5CpG$ sites. After incubation, samples were subjected to electrophoresis in gels for retardation assays. As the control, the same probe without methylation was used (Fig. 3). Results showed AtMBD4, AtMBD5, and AtMBD6 to form complexes with the methylated probe, whereas the other proteins were unable to bind under the conditions employed. However, control experiments showed that only AtMBD5 was specific to $m^5C$, being unable to bind the unmethylated probe, whereas AtMBD4 and AtMBD6 bound to the probe regardless of the methylation (Fig. 3). The possibility of binding to $m^5C$ in CpNpG and CpNpN sequences was then examined using synthetic oligomers (Fig. 4). The results clearly showed that AtMBD5 bound $m^5CpNpN$ but not $m^5CpNpG$, whereas AtMBD4 and AtMBD6 bound both probes (Fig. 4). Other AtMBDs did not bind to any of the probes. The specificity of AtMBD5 toward $m^5CpG$ and $m^5CpNpN$ was confirmed by competition, where a 50-fold excess of unlabeled methylated competitor effectively interfered with the binding, whereas the same amount of unmethylated DNA did not (Fig. 5). A similar competition assay showed neither AtMBD4 nor AtMBD6 to specifically target $m^5C$ (data not shown). Although the possibility remains that results obtained with recombinant

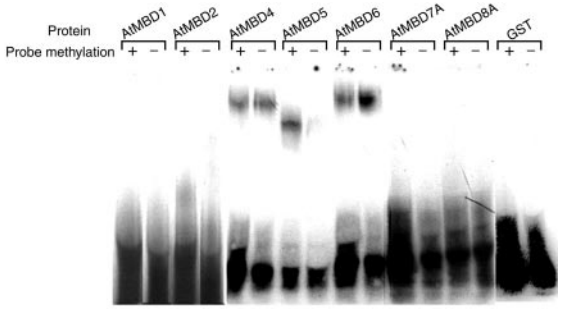


**Figure 3.** Binding assay of recombinant AtMBD proteins. Each AtMBD protein as indicated was prepared as a fused protein with GST in *E. coli*, purified, and incubated in a reaction mixture containing 50 ng of $^{32}P$-labeled 50-bp DNA fragment, in which cytosines were either methylated (+) or unmethylated (−) at CpG sites. After termination, the reaction mixture was subjected to 5% (w/v) polyacrylamide gel electrophoresis, and gels were subjected to autoradiography. As the control, GST protein was subjected to the same procedure.

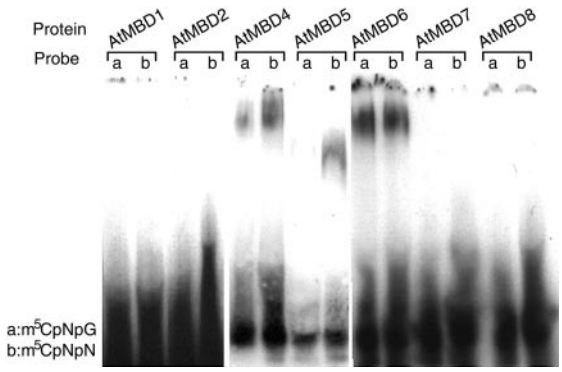


**Figure 4.** Binding to $m^5C$ in CpNpG and CpNpN sequences. A 50-ng synthetic oligonucleotide containing $m^5C$ in CpNpG (a) or CpNpN (b) was radioactively labeled with $^{32}P$ and incubated with 2 $\mu$g of the indicated AtMBD proteins. The reaction products were subjected to 5% (w/v) polyacrylamide gel electrophoresis, and gels were subjected to autoradiography.

protein samples, lacking posttranslational modification, may not completely reflect in vivo situation, these experiments suggested that, among the seven AtMBD proteins, only AtMBD5 is able to recognize $m^5C$ and that AtMBD4 and AtMBD6 might be nonspecific DNA-binding proteins. AtMBD1, AtMBD2, AtMBD7A, and AtMBD8A apparently are unable to directly bind to DNA.

### Chromosome Localization of AtMBD5

Chromosomal localization of AtMBD5 was visually assessed by histochemical staining (Fig. 6). When maize cells were subjected to binding assay with the bacterially expressed GST-AtMBD5 protein, fluorescent signals of the secondary antibody against GST were clearly observed in nuclei of interphase cells (Fig. 6, A–D). This is particularly distinct in metaphase, showing clear signals along individual chromosomes (Fig. 6, A–D). For more detailed observation, extended DNA fibers from Arabidopsis cells were prepared and visualized with the fluorescent dye YOYO-1 (green; Fig. 6E). The same preparation was then subjected to binding to AtMBD5 and visualized by rhodamine fluorescence of the secondary antibodies used for chromosome staining (Fig. 6D). Signals were found to track along the DNA fibers (Fig. 6F). This pattern resembled that of indirect immunostaining of extended DNA fibers with anti-$m^5C$ antibodies (Fig. 6G). Thus, these experiments indicated that AtMBD5 recognizes and binds to $m^5C$ in chromosomes.

### Tissue-Specific Localization

Accumulation of *AtMBD5* transcripts was examined by RNA-blot hybridization analysis. Total RNAs were isolated from leaf, stem, flower, or silique and subjected to hybridization with the cDNA probes for *AtMBD5*. Transcripts were found in all tissues examined, especially in flowers (Fig. 7A). Tissue-specific accumulation of AtMBD5 protein was then examined using polyclonal anti-AtMBD5 antibodies (Fig. 7B). The crude extracts were subjected to SDS-PAGE, and AtMBD5 protein with a molecular mass of 18 kD was detected by western-blot hybridization. AtMBD5 was shown to be almost ubiquitously distributed, although accumulation was undetectable in roots. The protein was found to be relatively abundant in seedlings.

### Expression of AtMBD5

To further analyze the precise location of AtMBD5 at both intracellular and organ levels, a chimeric construct of genomic DNA of AtMBD5 and the GFP gene was introduced into Arabidopsis. In the transgenic plants, accumulation of fusion protein could be regulated by the promoter and splicing of the AtMBD5 gene itself. Thus, localization of GFP can be considered to reflect the localization of AtMBD5 protein. As a reference, the fused construct driven by the CaMV 35S promoter was also used. The $T_3$ progeny of both transgenic lines was analyzed for GFP fluorescence in various parts (Fig. 8). When the authentic promoter was used, AtMBD5 was found to be exclusively expressed in the nuclei in root meristem cells at tips (Fig. 8A). This is a clear contrast to the control, in which *AtMBD5* was driven by the CaMV 35S promoter, showing GFP signals all over the root cells (Fig. 8B). In both cases, however, GFP fluorescence was observed in nuclei, consistent with observations in onion epidermal cells (Fig. 2E).

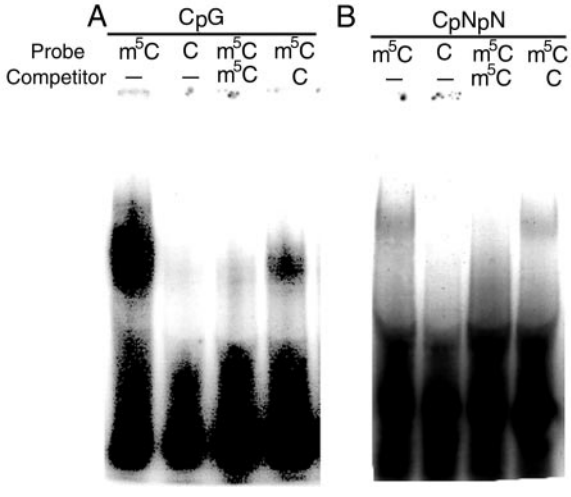


**Figure 5.** Competition assay. A 50-ng of synthetic oligonucleotide containing either cytosine (C) or $m^5C$ ($m^5C$) in CpG (A) or in CpNpN (B) was radioactively labeled and incubated with 2 $\mu$g of AtMBD5 in the presence or absence of 2.5 $\mu$g of unlabeled nonmethylated (C) or methylated ($m^5C$) competitor. As the competitor, a 500-bp fragment of actin cDNA (A) or unlabeled probe DNA (B) was used as described in the text. Samples were then electrophoresed through a 5% (w/v) acrylamide gel and subjected to autoradiography.

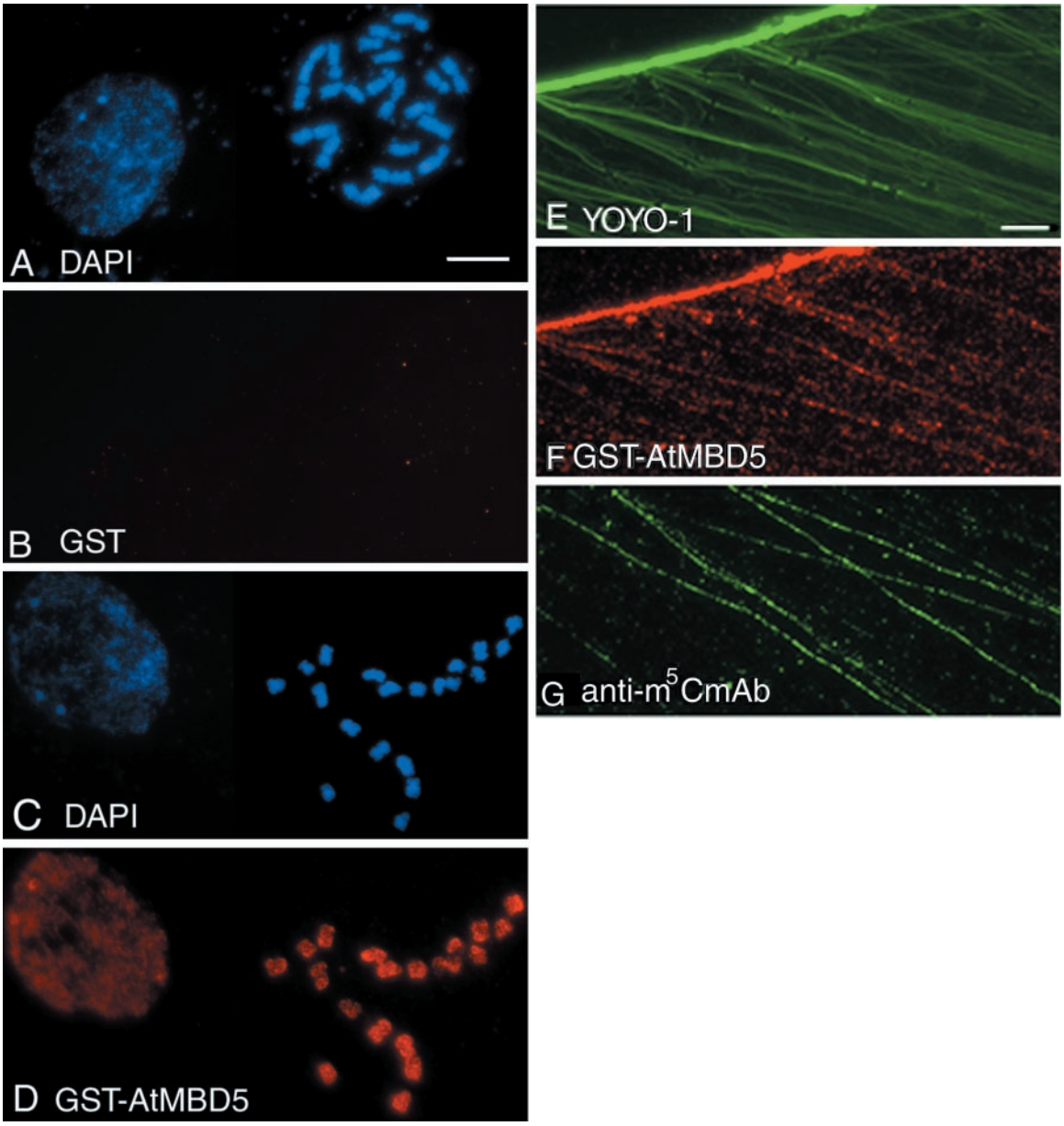


**Figure 6.** Histochemical analysis. Maize (*Zea mays*) nuclei and chromosomes were prepared on slide glasses and stained with 4′,6 diamidino-2-phenylindole (DAPI; A and C). Nucleus at interphase and metaphase is seen at left and right side, respectively. Samples were then subjected to binding with GST (B) or AtMBD5-GST fusion protein (D) and visualized with rhodamine-labeled anti-GST antibodies. Extended DNA fibers were simultaneously visualized with YOYO staining (E) and AtMBD5-GST fusion protein (F). A separately prepared extended DNA fiber was stained with monoclonal anti-$m^5C$ antibodies and visualized with the labeled secondary anti-mouse antibodies (G). Bar = 10 $\mu$m.

GFP fluorescence was strong in pistils (Fig. 8C), again showing a clear contrast to the control, with which GFP spread all over the flower organs (Fig. 8D). GFP signals were also observed in young seeds (Fig. 8E), and, upon more detailed observation, heart embryos were shown to be the dominant sites of expression (Fig. 8F). These observations indicate that AtMBD5 is predominantly expressed in actively proliferating cells and that it is present at a certain level even in quiescent mature tissues.

## DISCUSSION

Our search of the plant database for chromatin proteins identified 12 putative AtMBD proteins having similarity with human MeCP2, we have isolated corresponding cDNAs for seven of them. The encoded proteins were established to be diverse in size and in amino acid sequence, showing rather low homology to each other even in the MBD regions. However, a comparative analysis indicated that

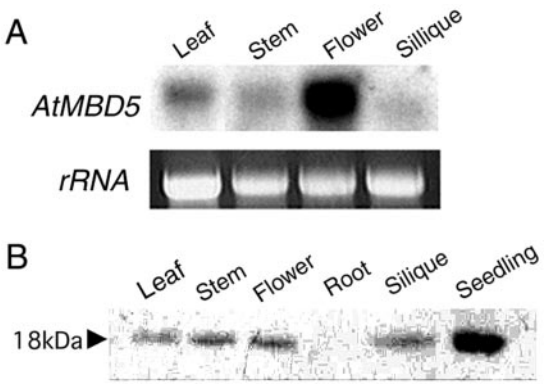


**Figure 7.** Tissue-specific localization. A, Transcript accumulation. Total RNA was extracted from the indicated tissues, and a 10-$\mu$g aliquot per lane was fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and subjected to successive hybridization with the *AtMBD5* probes. As an internal standard, rRNA stained with ethidium bromide is shown. B, Immunoblot analysis. Forty micrograms of total soluble proteins was extracted from indicated tissues and fractionated by 8% (w/v) SDS-PAGE. After blotting onto a cellulose membrane, AtMBD5 (18 kD) was identified by anti-AtMBD5 antibodies.

amino acids that were conserved in mammalian MBDs were also relatively well conserved in AtMBDs (Wakefield et al., 1999; Ohki et al., 2001). For example, Tyr-123 in MeCP2, which was suggested to interact directly with $m^5C$, was found to be well conserved among AtMBDs. Arg-111 and Arg-133 in MeCP2 are considered to interact with guanine bases in $m^5CpG$ and Asp-121 to form an electrostatic interaction with these arginines. These three amino acids are also conserved in AtMBDs, except in AtMBD8A.

Despite these common features, however, direct binding assays revealed that only AtMBD5 efficiently bind to $m^5CpG$ and $m^5CpNpN$. The molecular basis for such a specificity is not clear, but in addition to the conserved amino acids, amino acids flanking the mammalian MBD and the tertiary structure of the protein may be critical for $m^5CpG$ recognition and binding (Wakefield et al., 1999; Ohki et al., 2001). Mammalian MBD3 was shown not to be able to bind $m^5CpG$, despite all conserved amino acids being available (Hendrich et al., 2001). Our results are consistent with this observation and provide support for the conclusion that not all AtMBDs are functional for $m^5CpG$ binding. This raises the question as to whether AtMBDs bind to $m^5C$ in sequences other than CpG because in plant DNA, cytosines in CpNpG and CpNpN are frequently methylated (Oakeley and Jost, 1996). Our analyses revealed that none of the AtMBDs recognize $m^5CpNpG$ and that only AtMBD5 recognizes $m^5CpNpN$. AtMBD4 and AtMBD6 appeared to generally bind to DNA regardless of the methylation status.

Six AtMBDs have been detailed recently with regard to their $m^5C$-binding activity (Zemach and Grafi, 2003). AtMBD1, 2, and 4 through 7 were bacterially expressed as fusion proteins with GST and subjected to gel mobility assay with synthetic oligonucleotides. Among these six proteins, AtMBD5, 6, and 7 exhibited binding to $m^5CpG$ but not to $m^5CpNpG$. The results are essentially consistent with our observation that AtMBD5 is $m^5C$ specific. The discrepancy in results for other forms may be due to differences in the assay system because of the considerably diversity in protein preparation and concentration, composition of the reaction mixture, and substrate DNA. Nevertheless, it is clear from the available findings that not all AtMBDs recognize and bind to $m^5C$, raising further questions as to their biological function.

In plants, symmetric methylation of CpG is considered to maintain the methylation pattern through cell division and to globally regulate gene expression (Finnegan et al., 1998). Methylation in symmetric CpNpG also frequently occurs, the pattern of which is maintained through cell division, and involvement in repression of retrotransposons has been proposed (Lindroth et al., 2001). In contrast, methylation of asymmetric CpNpN is temporal, and its pattern is usually not transmittable (Oakeley and Jost, 1996). One of its functions is proposed to be participation in

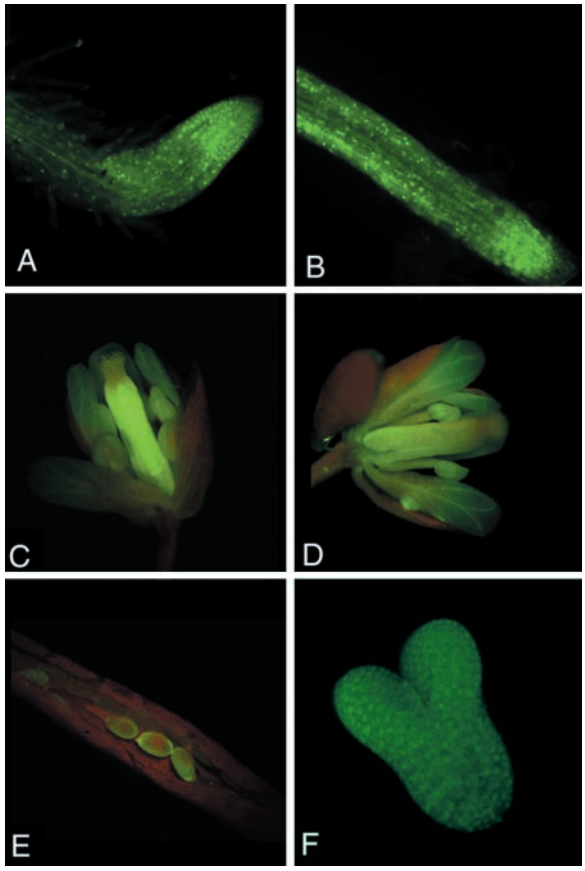


**Figure 8.** Tissue-specific expression. GFP-AtMBD5 fusion proteins driven by the *AtMBD5* authentic promoter (A, C, E, and F) or by the CaMV 35S promoter (B and D) were examined in root tissues (A and B), whole flowers at a young stage (C and D), developing seeds (E), and heart embryos (F).

transcriptional gene silencing (Jones et al., 2001). Because AtMBD5 specifically binds to $m^5C$ in CpG and CpNpN sequences, it also may be involved in regulation of gene expression and/or silencing. Because AtMBD5 is expressed not only in quiescent cells but also in floral organs and root tips, where cells actively proliferate, it is conceivable that AtMBD5 binds to $m^5C$ in newly synthesized DNA, thereby participating in remodeling of chromatin, as suggested from studies of mammalian counterparts.

In the case of mammalian MBD2 and MBD3, binding to $m^5CpG$ was found only in MBD2 but not in MBD3, although the latter forms a complex with histone deacetylase (NuRD; Hendrich et al., 2001). It was suggested that MBD3 is a key component of a corepressor complex of transcription, whereas MBD2 may serve in recruiting the former to DNA (Hendrich et al., 2001). Because AtMBD5 is the only protein that recognizes $m^5C$ among the seven AtMBDs, it is tempting to speculate that it serves as a bridge between methylated DNA and other proteins, including AtMBDs, as do mammalian MBDs. Whether or not AtMBD5 forms a complex with other AtMBDs and/or histone deacetylases now needs to be determined.

In summary, our findings for AtMBD5 raise many questions as to the functional mode of DNA methylation in plants. What proteins form complexes with AtMBD5? How do they interact with methylated DNA? What functions do other AtMBDs possess? What is the recognition mechanism for differential methylation in symmetric and asymmetric sequences? Currently, the physiological function of AtMBDs can only be speculated from knowledge obtained with mammalian systems. However, plant MBDs must possess inherent role(s), for which further study of AtMBD5 should provide valuable information.

## MATERIALS AND METHODS

### Plant Materials

Arabidopsis (ecotype Columbia) was grown on soil in a plastic tray at 21°C under long-day conditions (16-h-light/8-h-dark cycle) at a photon flux rate of 70 $\mu$mol $m^{-2}$ $s^{-1}$. Maize (*Zea mays* cv Golden Arrow) plants were hydroponically grown in a one-fifth strength Murashige and Skoog medium (Nihon Seiyaku, Tokyo) under continuous light for 13 d at 23°C and 70% relative humidity in a growth cabinet.

### cDNA Isolation

Arabidopsis genes encoding putative MBD proteins were identified in the plant chromatin database (http://chromdb.biosci.arizona.edu/): MBD1 (At4g22745), MBD2 (At5g35330), MBD3 (At4g00416), MBD4 (At3g63030), MBD5 (At3g46580), MBD6 (At5g59380), MBD7A (At5g59800), and MBD8A (At1g22310). Primers according to 5′ and 3′ ends of each putative open reading frame were synthesized and used for PCR with a mixture from a cDNA library constructed in HybriZAP (Stratagene, La Jolla, CA). PCR products were cloned into the TOPO II vector (Invitrogen, Carlsbad, CA) and sequenced using a DNA sequencing kit (Applied Biosystems, Foster City, CA) and a DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems).

### Construction and Expression of Fusion Proteins

The cDNA clones (*AtMBD1* through *AtMBD8A*, except for *AtMBD3*) were inserted into the GST fusion vector pGEX-4T-2 (Pharmacia Biotech, Uppsala), and *Escherichia coli* (DH5$\alpha$) was transformed with the resulting plasmids. Expression of GST fusion proteins was induced with 0.5 mM isopropyl-$\beta$-D-thiogalactoside at 20°C for 16 h. Cell samples were resuspended in 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 5 mM dithiothreitol, sonicated, and centrifuged to collect supernatants. Purification from the lysates was performed with a glutathione-Sepharose 4B column (Pharmacia Biotech) as indicated by the manufacturer. We have observed frequently that when the protein preparation became old or was repeatedly subjected to freezing-thawing, the binding specificity was lost, showing no binding activity or nonspecific DNA-binding feature. To avoid this, we always prepared fresh samples, which were used immediately after purification through the column.

### RNA-Blot Hybridization Analysis

Total RNA was extracted by the acid guanidinium thiocyanate-phenol chloroform method (Chomczynski and Sacchi, 1987). A 10-$\mu$g aliquot per lane was fractionated on a 1% (w/v) agarose gel containing 2% (v/v) formaldehyde, capillary blotted onto a nylon membrane (Hybond N, Amersham Biosciences, Piscataway, NJ) with 20× SSC, and fixed with UV light radiation. Each hybridization probe for the seven AtMBDs was labeled with [$\alpha$-$^{32}$P]dCTP using a DNA labeling kit (BcaBEST labeling kit, Takara, Otsu, Japan). Hybridization was carried out as described (Yoda et al., 2002). After hybridization, membranes were used to expose x-ray films.

### Epifluorescence Assay

The entire coding region of each AtMBD fragment was amplified by PCR using specific primers containing *Sal*I and *Nco*I sites and subcloned into the CaMV35S-sGFP(S65T)-NOS3′ vector so that fusion proteins of GFP and AtMBD were produced under the control of the CaMV 35S promoter. Cells in the epidermal layers of onion (*Allium cepa*) bulbs were transformed by particle bombardment as described (Yoda et al., 2002) with gold particles (Bio-Rad, Hercules, CA) coated with each plasmid. The control was CaMV35S-sGFP (S65T) alone. After bombardment, samples were incubated at 25°C in darkness for 12 h and viewed under epifluorescence microscopy (Ax70, Olympus, Tokyo).

### DNA-Binding Assay

For binding assays, the sequence containing five $m^5Cs$ in CpG was 5′-GCTCGTAGCTAACGAGCTCGACTCGTTGACATAGGCCATGGCGTAGACTC-3′ and that in CpNpG was 5′-GCTCTGAGCTAACAGGCTCAGCTCTGTGACATAGGCCATGGCTGAGACTC-3′ ($m^5C$ is indicated by the underline). Each complementary strand was also synthesized with $m^5C$ at symmetrical positions. The sequence containing five $m^5Cs$ in asymmetric CpNpN was 5′-GCTCTTAGCTAACAAGCTCAACTCTATGACATAGGCCATGGCTTAGACTC-3′, and its complementary strand was 5′-GAGTCTAAGCCATGGCCTATGTCATAGAGGTGAGCTTGTTAGCTAAGAGC-3′. Each oligonucleotide was annealed to form a duplex. Gel-shift assays were performed essentially as described (Yang et al., 2001) with modification. Double-stranded probe DNA was end labeled using [$\gamma$-$^{32}$P]ATP and $T_4$ polynucleotide kinase (New England Biolabs, Boston), and a 1-$\mu$L aliquot containing approximately $10^4$ cpm was mixed with approximately 2 $\mu$g of GST-AtMBD protein in a 15-$\mu$L reaction mixture containing 20 mM HEPES (pH7.9), 1 mM EDTA (pH8.0), 3 mM $MgCl_2$, 10 mM $\beta$-mercaptoetanol, 8% (w/v) glycerol, and 0.1% (w/v) Triton X-100. After incubation at room temperature for 20 min, the reaction mixture was subjected to electrophoresis on a 5% (w/v) polyacrylamide gel in 6.725 mM Tris-HCl (pH 7.9), 3.3 mM Na-acetate, and 1 mM EDTA (pH 8.0). Competition assays were performed with enzymatically methylated native DNA as described (Hendrich and Bird, 1998). In brief, a 500-bp fragment from Arabidopsis actin cDNA was amplified by PCR and used as the competitor after methylation by SssI methylase. For CpNpN competition assay, the probe DNA without labeling was used at appropriate concentrations.

## Histochemical Observation

Maize root tips harvested from 3-d-old seedlings were fixed in a solution of acetic acid:ethanol (1:3 [v/v]) for 16 h. Chromosomes were prepared as described (Fukui and Iijima, 1991) with modification. Root tips cut 1 mm long were macerated in an enzyme solution containing 4% (w/v) cellulase Onozuka RS (Yakult, Tokyo) and 1% (w/v) pectolyase Y-23 (Seishin, Tokyo), adjusted to pH 4.2 at 37°C for 30 min, placed on a glass slide, tapped with the tips of fine forceps with a few drop of methanol:acetic acid (3:1 [v/v]) solution, and air-dried. Extended DNA fibers from leaves of Arabidopsis were prepared as described (Ohmido et al., 2001). Chromosomes and extended DNA fibers on the slides were subjected to binding under the same conditions as for the gel shift assay. Immunostaining was performed using goat anti-GST antibodies (Amersham Biosciences), diluted 1:100 (w/v) in phosphate-buffered saline and 0.05% (w/v) Tween 20 and anti-Goat-Cy3 antibodies (Sigma, St. Louis), similarly diluted, as the secondary antibody. Chromosomes and extended DNA fibers were counterstained in 1 $\mu$g mL$^{-1}$ DAPI and 1 $\mu$g mL$^{-1}$ YOYO-1 iodide (Molecular Probes, Eugene, OR)/Vectashieled solutions (Vector Laboratories, Burlingame, CA), respectively. The m$^5$C in DNA fibers was stained with an anti-m$^5$C mAb, and indirect immunofluorescence staining was performed as described (Nishiyama et al., 2002). Fluorescent images of DAPI, YOYO-1, and Cy3 were observed under a fluorescence microscope equipped with UV, B, and G excitation filters (Ax70, Olympus) and captured separately using a cooled CCD camera (CoolSNAP-HQ, Photometrics, Tucson, AZ).

## Immunoblot Analysis

Organ tissues were ground in liquid nitrogen in a mortar, mixed with isolation buffer containing 50 mM Tris-HCl (pH7.5), 100 mM NaCl, 0.05% (w/v) Tween 20, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 $\mu$g mL$^{-1}$ leupepin, 5 $\mu$g mL$^{-1}$ aprotinin, and homogenized. After centrifugation at 15,000 rpm for 10 min to remove insoluble debris, crude extracts were directly subjected to SDS-PAGE, and proteins were electronically transferred onto cellulose membranes (Immobilon-N, Millipore, Bedford, MA). AtMBD5 protein was detected with an anti-AtMBD antibody raised in rabbits.

## Construction of Transgenic Plants and Fluorescence Imaging

Genomic DNA of Arabidopsis amplified by PCR with primers (5′-GGATCCAAGACAGAAGGTTAAAAACATTGAG-3′ and 5′-ATTC-CATGGAGAACATCGTTTTTCCAGCGTCGTTTTG-3′) was fused with pblue-sGFP(S65T)-NOS SK using *Bam*HI and *Nco*I sites. A chimeric gene of AtMBD5 and GFP of the resulting plasmid were cut out with *Xba*I and *Sal*I and then replaced with the GUS gene of pBI10I. For this replacement, the *Sac*I site of pBI101 was converted to *Xho*I that is compatible with *Sal*I in the ligation. This construct was introduced into Arabidopsis by in plant infiltration, and transgenic plants were observed using a stereomicroscope equipped with a GFPII filter (MZFLIII, Leica, Wetzler, Germany) and captured using a digital camera (C4742 95, Hamamatsu Photonics, Hamamatsu, Japan).

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