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Methyl-CpG-binding domain proteins in plants: interpreters of DNA methylation

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The effect of DNA methylation on various aspects of plant cellular and developmental processes has been well documented over the past 35 years. However, the underlying molecular mechanism interpreting the methylation signal has only recently been explored with the isolation and characterization of the *Arabidopsis* methyl-CpG-binding domain (MBD) proteins. In this review, we highlight recent advances and present new models concerning *Arabidopsis* MBD proteins and their possible role in controlling chromatin structure mediated by CpG methylation.

DNA methylation: significance and occurrence

DNA methylation, a common epigenetic modification in the genome of plants and animals, is a powerful mechanism for regulating gene expression. Most methylation in the eukaryotic nuclear DNA occurs at position five of the pyrimidine ring of cytosine [1,2]. The biological significance of cytosine methylation in plants was deduced from its dynamics during development and pathogen infection as well as from its correlation with transgene silencing [2,3]. Furthermore, treatments with the hypomethylating agent 5-azacytidine as well as genetic manipulation of DNA methylation levels helped to unravel the crucial role of cytosine methylation in gene expression, genome organization and plant development [2,4–7]. In plants, most 5-methylcytosines are associated with heterochromatic, transcriptionally inactive regions, which are often enriched with repetitive DNA sequences. Yet, in *Arabidopsis thaliana*, the genome-wide high-resolution mapping of DNA methylation has recently revealed that over one-third of expressed genes contain methylations within the transcribed regions [8]. Whereas, in animals, cytosine methylation is prevalent in symmetrical CpG dinucleotides, in plants, it is often found in symmetrical CpG and CpNpG as well as in non-symmetrical CpHpH (H = C, A or T) contexts [9,10]. A growing body of evidence suggests that cytosine methylation at specific chromosomal domains is triggered by RNA molecules (i.e. small interfering RNAs) in a mechanism known as RNA-dependent DNA methylation (RdDM) [11–13].

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In spite of the intensive study of DNA methylation in plants, the way in which the methyl group is interpreted into basic cellular functions has only recently begun to be explored with the isolation and characterization of methylated DNA-binding proteins (MBP), namely, the *Arabidopsis* methyl-CpG-binding domain (MBD) proteins [14–17]. This review focuses on recent advances in our understanding of the mechanism through which DNA methylation is recognized and targeted for chromatin compaction and gene repression. We highlight the discrepancy between amino acid sequence homology and functionality (i.e. binding to methylated CpG sites) of MBD-containing proteins, as well as the divergence between monocot and dicot MBD proteins. Moreover, we emphasize similarities and differences between plant and animal MBD proteins and provide new speculative models that focus on plant MBD proteins-induced chromatin compaction and gene silencing.

MBD motif is conserved and common in *Arabidopsis*

Plant nuclear proteins capable of binding methylated cytosines *in vitro* have long been known. A plant nuclear protein designated DBPm capable of binding specifically 5-methylcytosine in any DNA sequence context was first described in pea [18]. Subsequently, similar proteins were characterized in a variety of plant species including corn, wheat and carrot [19,20]. In nuclear extracts of carrot (*Daucus carota*), two classes of MBPs were identified: (i) dcMBP1, which showed high affinity for 5-methylcytosine in CpG context, and (ii) dcMBP2, which showed high affinity for 5-methylcytosine in CpNpG and CpHpH contexts [20]; none of these MBPs have been isolated and characterized. Great progress in identifying plant MBP-encoding genes (i.e. MBD proteins) was made with the release of various plant genome and EST sequences. Based on amino acid sequence homology with animal MBDs (Box 1) (reviewed in Ref. [21]), the Plant Chromatin database (http://www.chromdb.org/) lists 13 genes encoding putative MBD proteins in *Arabidopsis*, 17 genes in rice (*Oryza sativa* subsp. *japonica*), 14 genes in maize (*Zea mays*) and 14 genes in poplar (*Populus trichocarpa*). Bioinformatics analyses of plant MBDs are presented in Refs [16,22]. The *Arabidopsis* gene family is divided into eight subclasses based on sequence

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Box 1. Mammalian MBD proteins

In animals, cytosine methylation occurs predominantly at CpG dinucleotide sequences, and plays a fundamental role in controlling various cellular and developmental processes (reviewed in Ref. [21]). The cloning of a gene whose product is capable of binding methylated CpG sites, namely MeCP2 [56], has provided insights into the underlying mechanisms by which the DNA methylation signal is interpreted into a functional state (i.e. chromatin compaction and gene silencing). The minimal domain possessing methyl-CpG-binding activity, termed methyl-CpG-binding domain (MBD), was found to consist of 85 amino acids [57]. The solution structure of the MBD motif of human MBD1 in complex with methylated DNA was resolved by NMR spectroscopy. This analysis revealed that recognition is due to five highly conserved amino acid residues that form a hydrophobic patch, which mediates the contacts with methyl-CpG dinucleotides [58]. In humans, there are five MBD proteins, named MBD1–MBD4 in addition to the founding member of the family, MeCP2; with the exception of MBD3, all these proteins specifically recognize and bind methylated CpG sites. The mammalian MBD4 represents a unique protein that has a thymine glycosylase activity, which mediates G:T mismatch repairs often found in methylated CpG regions [59]. The human genome encodes six additional proteins possessing a putative MBD motif, termed TAM (TIP5/ARBP/MBD) [21]. Based on amino acid residue analysis, these proteins are predicted to be non-functional with regard to their binding of methylated CpG sites [21]. The biological significance of MBD proteins is demonstrated in the Rett syndrome (after Andreas Rett, an Austrian physician who first described this disorder in 1966), which is an X chromosome-linked childhood neuro-developmental disorder resulting from mutations in the gene encoding the MBD transcriptional repressor MeCP2 (reviewed in Ref. [60]).

similarity within the MBD motif. Although a relatively high degree of amino acid sequence conservation is found between *Arabidopsis* and human MBD motifs [14,22], no other sequence homologies exist between those proteins. Furthermore, no homology has been found between the *Arabidopsis* proteins and transcription repression domain (TRD) sequences found in animal MBDs [23]. As in the case of mammalian MBDs, most *Arabidopsis* MBD (AtMBD)-encoding genes contain an intron within the MBD motif [14]. Phylogenetic analysis of the aligned MBD motif sequences shows the highest similarity between AtMBD5, AtMBD6 and AtMBD7 and the human functional MBD motifs [14,22]; AtMBD7, which contains three MBD motifs, represents a unique MBD protein not found in animals. AtMBD5 and AtMBD6 might be functionally redundant because they share high amino acid sequence homology along the entire molecule except for the additional 54 amino acids found at the N-terminus of AtMBD6. Based on sequence similarity and intron position, AtMBD5, AtMBD6 and AtMBD7 have been suggested to originate from a common ancestor [16]. Interestingly, bioinformatics analysis points to the evolutionary divergence of dicot MBD proteins from those of monocots because subclasses IV (AtMBD5 and AtMBD6) and VI (AtMBD7) appear to be unique to dicots [22].

There is some controversy regarding the ability of AtMBD proteins to bind methylated CpG sites *in vitro* (Table 1). However, the general consensus seems to be that of the AtMBD proteins examined so far, only AtMBD5, AtMBD6 and AtMBD7 are capable of binding specifically methylated CpG sites *in vitro* (these proteins are referred to as functional MBD proteins), whereas AtMBD1, AtMBD2, AtMBD4, AtMBD8 and AtMBD11 are not [14,15,17]. In addition, AtMBD5 has also been reported to bind 5-methylcytosine in a CpHpH context [15,17], whereas AtMBD4 and AtMBD11 bind DNA in a methylation-independent manner [15,17]. AtMBD3 and AtMBD12 are likely to be pseudogenes [16], and the MBD motif of AtMBD13 only shares a partial and limited homology with functional MBD motifs [22]. AtMBD1, AtMBD2 and AtMBD4 possess a CW-type zinc finger domain (zf-CW) found in nuclear proteins of vertebrates

Table 1. Summary of *in vitro* methyl cytosine-binding and cellular distribution of AtMBD-containing proteins

Gene	Binding to or context of methylcytosine[a]	Refs	Cellular distribution[b]	Refs
AtMBD1 (AT4G22745)	No	[14,15,17]	Onion, nuclear	[17]
AtMBD2 (AT5G35330)	No	[14,15,17]	Onion, nuclear	[17]
			Arabidopsis, nuclear, excluding chromocentres	[26]
AtMBD3 (AT4G00416)	ND		ND	
AtMBD4 (AT3G63030)	No	[14,15]	Onion, nuclear	[17]
	Yes, CpG; CpNpG; CpHpH (not specific)	[17]		
AtMBD5 (AT3G46580)	Yes, CpG	[14,15]	Onion, nuclear	[17]
	Yes, CpHpH	[15,17]	*Arabidopsis*, mainly chromocentres	[15,26]
AtMBD6 (AT5G59380)	Yes, CpG	[14,15]	Onion, nuclear	[17]
	Yes, CpNpG; CpHpH (not specific)	[17]	*Arabidopsis*, mainly chromocentres	[15,26]
AtMBD7 (AT5G59800)	Yes, CpG	[14]	Onion, nuclear	[17]
	No	[17]	*Arabidopsis*, mainly chromocentres	[26]
AtMBD8 (AT1G22310)	No	[17]	Onion, nuclear	[17]
AtMBD9 (AT3G01460)	ND		ND	
AtMBD10 (AT1G15340)	ND		ND	
AtMBD11 (AT3G15790)	Yes, CpG; CpNpG; CpNpN (not specific)	[15]	Onion, nuclear	[16]
AtMBD12 (AT5G35338)	ND		ND	
AtMBD13 (AT5G52230)	ND		ND	

Abbreviation: ND, not determined.
[a]CpHpH (H = C, A or T); CpNpG (N = any nucleotide).
[b]The localization in onion refers to transient expression of the *Arabidopsis* MBDs in onion cells.

and higher plants, which is predicted to play a role in DNA binding and/or protein–protein interactions [24].

To date, the methylcytosine-binding specificity of the monocot MBD proteins has not been tested. The finding that monocots lack homologues of functional MBD proteins (subclasses IV and VI) [22] casts doubt on the relevance of monocot MBD proteins in interpreting the cytosine methylation signal. It is conceivable that MBD-containing proteins in monocots have become highly diverged throughout evolution and might carry out functions other than that of methylated CpG sites-binding such as binding to RNA or to unmethylated DNA [15,17,25], and/or that the MBD motif might provide a surface for protein–protein interactions [25,26]. The DNA methylation signal in monocots might, therefore, be interpreted by another subclass of the MBD family, or by group of proteins as yet uncharacterized (e.g. Kaiso-like proteins [27,28]).

In vivo localization of AtMBDs fused to GFP in *Arabidopsis* nuclei revealed colocalization of AtMBD5, AtMBD6 and AtMBD7 to the highly methylated chromocentres (Figure 1) [15,26], solving previous controversy regarding their capability to bind *in vitro* methyl-CpG sites

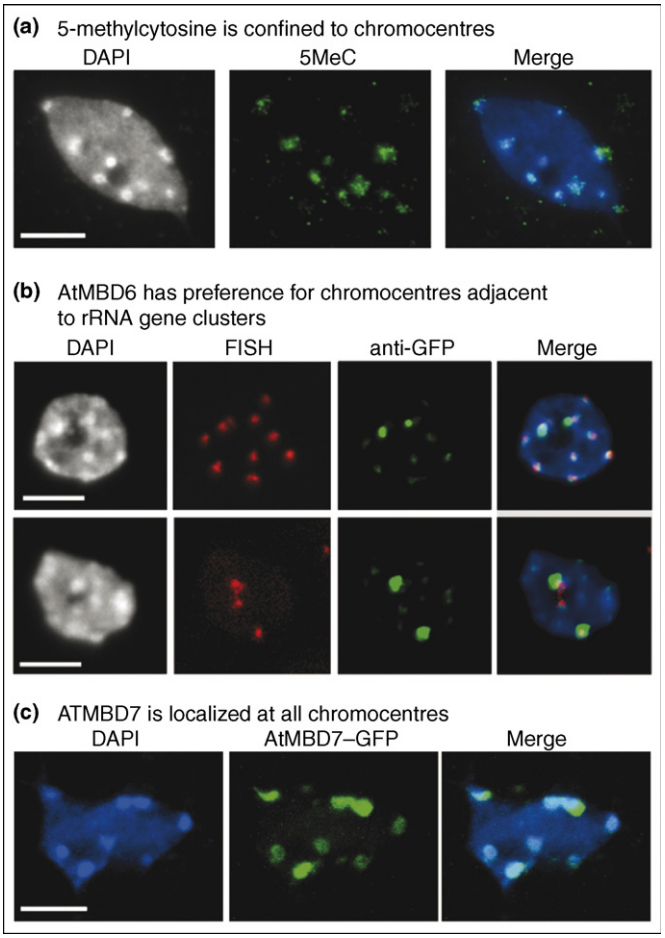

Figure 1. Functional AtMBD proteins are localized at highly methylated chromocentres. **(a)** Immunolabelling demonstrating that 5-methylcytosines are concentrated at and around the intensely DAPI-stained chromocentres. **(b)** Immunolabelling and fluorescence *in situ* hybridization demonstrating the preference of AtMBD6-GFP for chromocentres adjacent to rRNA gene clusters. Part (b) reproduced, with permission, from Ref. [26]. **(c)** Transient expression of AtMBD7–GFP in *Arabidopsis* protoplasts showing its localization at all chromocentres. Scale bars = 5 μm. Abbreviation: 5MeC, 5-methylcytosine; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization.

[14,15,17]. Indeed, CpG methylation is indispensable for the localization of these AtMBDs to chromocentres inasmuch as their nuclear localization is disrupted in the DNA hypomethylation mutants *ddm1* and *met1* [26], as well as in cells treated with the hypomethylating agent 5-azacytidine [15]. These attributes are also found in mammalian MBD proteins, which localize mainly in nuclear foci enriched in methyl CpG. In mouse cells deficient in CpG methylation, most MBDs are dispersed within the nucleus [29]. Consistent with this observation, AtMBD2 and AtMBD11, which are unable to bind specifically methylated CpG sites *in vitro*, also show dispersed distribution within the nucleus [15,17,26]. Yet, AtMBD2 has a peculiar distribution pattern in *ddm1* and *met1* backgrounds, where it accumulates at chromocentres [26]. This supports the notion that AtMBD2 binds chromatin tightly but independently of cytosine methylation [26]. The mechanism(s) underlying AtMBD2 recruitment to the smaller chromocentres of these hypomethylated mutants (*ddm1* and *met1*) is unknown. Plausibly, the core of heterochoromatic chromocentres in these mutants is preserved owing to the recruitment of proteins or protein complexes capable of maintaining compact chromatin configuration in the absence of DNA methylation. This hypothesis is supported by the finding that the human MeCP2, the first member of the human MBD protein family to be identified (Box 1), can induce compaction of chromatin in the absence of DNA methylation [30].

AtMBD5 and AtMBD6 are likely to participate in rRNA gene silencing

An apparent difference has been noted between the localization of AtMBD7 and that of AtMBD5 and AtMBD6. Whereas AtMBD7 frequently localizes to all chromocentres, AtMBD5 and AtMBD6 show a preference for two perinucleolar chromocentres adjacent to ribosomal DNA (rDNA) gene clusters (Figure 1b) [26]. This preference suggests a role for AtMBD5 and AtMBD6 in silencing of rDNAs. In animals, the nucleolar remodelling complex (NoRC) induces rDNA silencing by recruiting DNA methyltransferases and histone deacetylase (HDAC) activity to rDNA promoter regions [31]. NoRC consists of SNF2h, a chromatin remodelling factor, and TIP5, a nucleolar protein that possesses an MBD motif. Likewise, AtMBD6 has been shown to interact with protein complexes containing HDAC activity [14] known to be required for rRNA gene silencing in plants; thus, HDT1 (HD2 family) and HDA6 (RPD3 family) histone deacetylases have been shown to be involved in switching off transcription of rRNA genes in *Arabidopsis* [32–34].

The preference for perinucleolar chromocentres further suggests that binding of AtMBD5 and AtMBD6 to these sites is not solely dependent on cytosine methylation but on additional factors, such as specific DNA sequences and/or specific proteins such as DDM1. Indeed, the MeCP2 protein (Box 1) has recently been found to have a preference for methylated CpG sites adjacent to several A/T dinucleotide repeats [35], and the human MBD2 has been shown to bind and repress specific target genes [36]. The binding to perinucleolar chromocentres might be mediated by interaction with DDM1, previously shown to bind

to AtMBD5 and AtMBD6 *in vitro* [26]. However, the localization of these proteins is disrupted in the *met1* mutant, which is deficient in DNA methylation but has an active form of DDM1 [26], suggesting that cytosine methylation is indispensable for AtMBD5 and AtMBD6 localization at perinucleolar chromocentres. However, the question remains as to whether AtMBD5 and AtMBD6 bind to CpG methylated sites directly or via an interaction with an unidentified factor(s).

AtMBDs and plant development

Expression assays show that all tested *AtMBD* genes have detectable steady-state levels of transcript in all tissues examined, although levels of expression can vary between tissues [14–17]. For example, *AtMBD5* shows higher expression in reproductive organs compared with that of *AtMBD6* [15,17]. Therefore, a common tissue- and developmental stage-specific regulation of functional *AtMBD* genes is not apparent. Downregulation of *AtMBD6* and *AtMBD7* gene expression by T-DNA insertion does not reveal any phenotypes (A. Zemach and G. Grafi, unpublished) suggesting functional redundancies between AtMBD proteins. Therefore, it will be necessary to generate the relevant triple mutant to evaluate the importance of functional AtMBDs in genome organization and plant development.

To date, only AtMBD11 and AtMBD9, both of which are likely to be non-functional MBDs, have been found to be involved in plant development. Downregulation of AtMBD11 by RNA interference leads to developmental defects including late flowering and reduced fertility [16]. A T-DNA insertion mutation in the *AtMBD9* gene has been shown to increase shoot branching and leads to transcriptional repression of the *FLOWERING LOCUS C* (*FLC*) gene and, consequently, to early flowering [37]. This observation suggests that AtMBD9 is involved in gene activation rather than gene repression, further supporting the notion that its MBD motif is non-functional regarding binding to methylated CpG dinucleotides [16]. This is similar to mammalian TAM proteins (Box 1) whose MBD motifs are predicted to be non-functional [21]. AtMBD9 possesses multiple motifs thought to be involved in chromatin-mediated gene regulation. These include PHD (plant homeodomain) finger domains found in proteins participating in chromatin remodelling complexes [38], and a bromodomain, an acetyl lysine-binding domain found in chromatin-associated proteins and in nearly all histone acetyltransferases involved in transcriptional activation [39].

How do AtMBD proteins induce formation of repressive chromatin?

In mammalian cells, multiprotein repressive complexes consist of MBD proteins, SWI/SNF2 chromatin-remodelling factors and HDACs [40,41]. The current model suggests that, following their binding to methylated CpG sites, MBD proteins recruit various enzymes that chemically modify core histone proteins and, hence, affect the local chromatin structure [42]. An interplay between cytosine methylation and the methylation of lysine 9 of histone H3 (H3K9) at silent chromatin regions has been reported in *Neurospora crassa* and *Arabidopsis* [43–45]. The accumulated data indicate that the epigenetic signalling leading to methylation of cytosine or that of histone H3 (at lysines 9 and 27) is non-linear and each epigenetic mark (i.e. cytosine methylation or histone methylation) is established either dependently or independently of the other epigenetic mark [46–49]. The dependency of H3K9 methylation on CpG methylation, and vice versa, can be mediated by MBD proteins. Indeed, MeCP2 has been found to interact with histone methyltransferase and induce H3K9 methylation [50]; moreover, human MBD1 has been shown to interact with the Suv39h1-HP1 heterochromatic complex and to induce DNA methylation-based transcriptional repression [51]. Furthermore, during DNA replication, MBD1 recruits the H3K9 methylase SETDB1 to the large subunit of the chromatin assembly factor CAF-1, thereby facilitating methylation of H3K9 [52]. *Arabidopsis* MBD protein complexes might share common features with mammalian MBD complexes, such as the SWI2/SNF2 chromatin-remodelling factor DDM1 and the

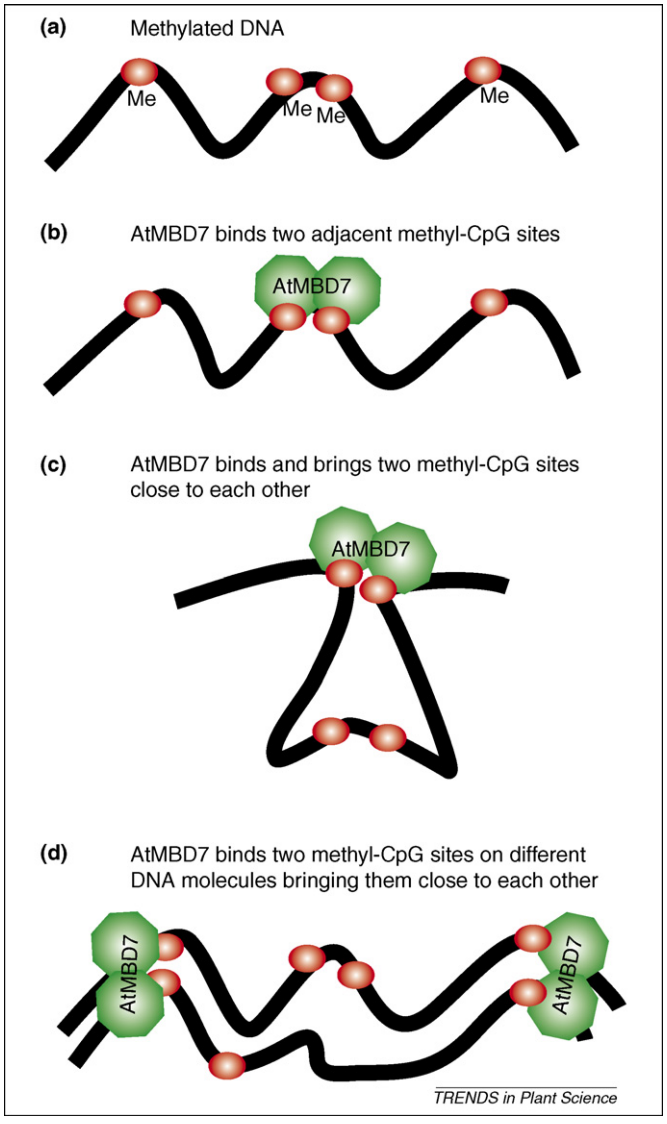

Figure 2. Chromatin compaction mediated by AtMBD7. The figure depicts several potential ways for binding of AtMBD7 to methyl-CpG sites using its two functional MBD motifs. Abbreviation: Me, methylated CpG site.

association with HDAC activity [14,26], linking DNA methylation with histone modifications. Hence, like their animal counterparts, AtMBDs might affect chromatin structure by inducing modifications of core histone proteins at methylated CpG domains. For example, methylation of H3K9 and H3K27 generates binding sites for chromatin modifiers such as LIKE HETEROCHROMATIN PROTEIN1 (LHP1) [44,53] or CHROMOMETHYLASE3 (CMT3) [54] leading to chromatin reorganization.

However, plants possess a unique class of MBD proteins that is not found in animals, the members of which have several MBD motifs. The Plant Chromatin Database (http://www.chromdb.org/) lists one such protein in *Arabidopsis* (MBD7; referred to as AtMBD7 in this article), one in poplar (MBD914), one in maize (MBD114) and five proteins in rice (MBD704, MBD705, MBD712, MBD714 and MBD716). In *Arabidopsis*, for example, AtMBD7 contains three MBD motifs, two of which bind methylated CpG sites *in vitro* [14]. Consequently, one molecule of AtMBD7 is predicted to bind at least two methylated CpG sites. This might constitute a novel mechanism for MBD-induced heterochromatin formation. Indeed, having several methylated CpG-binding sites, AtMBD7 might be able to bring together DNA sequences that are not adjacent and maintain them close to one another (Figure 2), thus promoting and/or maintaining a compact configuration.

Concluding remarks

The presence of an MBD motif does not necessarily point to methyl-CpG-binding activity. Whereas the *Arabidopsis* proteins AtMBD5, AtMBD6 and AtMBD7 have been proven to bind methyl CpG *in vitro* and to localize to highly methylated chromocentres *in vivo* (and are thus referred to as functional MBD proteins), none of the other *Arabidopsis* MBD-containing proteins show these properties. Therefore, the majority of the *Arabidopsis* MBD-containing proteins are non-functional MBDs, as is the case for animal TAM proteins (Box 1). One can speculate that a functional MBD motif might represent the ancestral MBD motif and that the non-methyl CpG-binding MBD motifs might have evolved other functions, such as binding to RNA or unmethylated DNA [15,17,25] and/or provide surfaces for protein–protein interactions [26,55]. Alternatively, a functional MBD motif could have arisen from a non-functional one, or from another as yet unknown, structurally related motif.

The functional MBDs, namely, AtMBD5, AtMBD6 and AtMBD7, emerge as possible mediators of the CpG methylation-induced formation of repressive chromatin. The mechanisms underlying the function of these proteins have begun to unravel with the finding that they interact with the protein DDM1 and their association with HDAC activity. Plants, unlike animals, possess a unique class of MBD proteins that contain two or three MBD motifs. This feature might be mechanistically relevant for chromatin compaction. A greater understanding of the mechanisms involved in MBD function could be achieved through: (i) the identification of proteins that physically interact with MBD proteins; (ii) the isolation and characterization of multiprotein complexes associated *in vivo* with MBD proteins; (iii) studying mutants in genes encoding for functional MBD proteins; and (iv) unravelling the capability of the unique group of plant MBD proteins containing two or more functional MBD motifs to directly affect chromatin compaction (Figure 2).

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