The Methyl-CpG-Binding Protein MBD7 Facilitates Active DNA Demethylation to Limit DNA Hyper-Methylation and Transcriptional Gene Silencing

Graphical Abstract

Highlights
- MBD7 and IDM3 prevent DNA hypermethylation and gene silencing
- MBD7 binds to genomic regions with a high density of CG methylation
- MBD7 prevents DNA methylation spread
- MBD7 physically associates with IDM3, and with IDM1 and IDM2

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In Brief
Lang et al. show that MBD7 binds to highly methylated, CG-dense chromatin regions and physically associates with IDM proteins to enable these DNA demethylases to prevent aberrant spreading of DNA methylation.

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The Methyl-CpG-Binding Protein MBD7 Facilitates Active DNA Demethylation to Limit DNA Hyper-Methylation and Transcriptional Gene Silencing

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SUMMARY

DNA methylation is a conserved epigenetic mark that plays important roles in plant and vertebrate development, genome stability, and gene regulation. Canonical Methyl-CpG-binding domain (MBD) proteins are important interpreters of DNA methylation that recognize methylated CG sites and recruit chromatin remodelers, histone deacetylases, and histone methyltransferases to repress transcription. Here, we show that Arabidopsis MBD7 and Increased DNA Methylation 3 (IDM3) are anti-silencing factors that prevent gene repression and DNA hypermethylation. MBD7 preferentially binds to highly methylated, CG-dense regions and physically associates with other anti-silencing factors, including the histone acetyltransferase IDM1 and the alpha-crystallin domain proteins IDM2 and IDM3. IDM1 and IDM2 were previously shown to facilitate active DNA demethylation by the 5-methylcytosine DNA glycosylase/lyase ROS1. Thus, MBD7 tethers the IDM proteins to methylated DNA, which enables the function of DNA demethylases that in turn limit DNA methylation and prevent transcriptional gene silencing.

INTRODUCTION

DNA methylation, a conserved epigenetic mark in plants and vertebrates, plays important roles in development, genome stability, and in several well-known epigenetic phenomena such as genomic imprinting, paramutation, and X chromosome inactivation (He et al., 2011; Law and Jacobsen, 2010; Tariq and Paszkowski, 2004). In mammals, 5-methylcytosine (5mC) occurs mainly at CG dinucleotides, whereas in plants, 5mC is found in three sequence contexts: CG, CHG, and CHH (where H is A, C, or T) (He et al., 2011; Law and Jacobsen, 2010). In plants, DNA methylation can be established through the RNA-directed DNA methylation (RdDM) pathway (Matzke and Mosher, 2014; Haag and Pikaard, 2011; Zhang and Zhu, 2011). Maintenance of CG and CHG methylation requires the Dnmt1 ortholog MET1, and the plant specific enzyme CMT3, respectively (He et al., 2011; Law and Jacobsen, 2010). CHH methylation is carried out by DRM2 through the RdDM pathway (Matzke and Mosher, 2014) and by CMT2, which requires the chromatin remodeling protein DDM1 (Zemach et al., 2013).

DNA methylation is associated with transcriptional silencing (He et al., 2011; Law and Jacobsen, 2010; Tariq and Paszkowski, 2004). Methyl-CpG-binding domain (MBD) proteins are important interpreters of DNA methylation (Hendrich and Bird, 1998). Generally, MBD proteins recognize methylated CG sites and recruit chromatin remodelers, histone deacetylases, and histone methyltransferases to repress transcription (Ng et al., 1999). Recent analysis of the genome-wide binding sites of MBD proteins in mammalian cells confirmed that MBD proteins are typically enriched at methylated genomic regions and methylation-dependent MBD protein binding at promoters coincides with gene silencing (Baubec et al., 2013). The Arabidopsis genome is predicted to encode 13 canonical MBD proteins, although only three of them, MBDS, MBDB, and MBD7, bind specifically to methylated CG sites in vitro (Zemach and Graﬁ, 2007). The functions of these plant MBD proteins are poorly understood, but the available information is consistent with a conserved role as effectors of DNA methylation that cause transcriptional repression (Zemach and Graﬁ, 2007).

DNA methylation is dynamically controlled by both methylation and demethylation reactions (Zhu, 2009). In vertebrates, active DNA demethylation is initiated by oxidation or deamination of...
5-methylcytosine (5mC) by TET enzymes or AID/APOBECs, respectively, followed by the DNA glycosylase TDG or MBD4 (Rai et al., 2008; Kohli and Zhang, 2013), whereas in plants, active DNA demethylation is initiated by the ROS1/DME family of 5mC DNA glycosylases (Zhu, 2009). In Arabidopsis, ROS1 is necessary for preventing DNA hypermethylation at thousands of genomic regions (Qian et al., 2012). How the DNA demethylation enzymes are targeted to specific genomic loci is poorly understood. At a subset of these genomic regions, ROS1 function required the histone acetyltransferase Increased DNA Methylation SUC2 (Lei et al., 2014; Wang et al., 2013). Further, these plants express alleles of known anti-silencing factors, such as IDM1 and Downy Mildew 2 (EDM2), and IDM2, in addition to new anti-silencing factors, such as IDM1 and ROS1 (Lei et al., 2014; Wang et al., 2013). We found that both MBD7 and IDM3 limit DNA methylation, particularly at transposable elements (TEs). MBD7 is enriched at highly methylated, CG-dense sites throughout the genome, and this enrichment coincides with its function in preventing DNA methylation spread or hypermethylation. Protein interaction analyses revealed that MBD7 associates with ID2M and IDM3 (an IDM2-like protein) in vitro and in vivo and also associates with IDM1 in vivo. These results suggest a model in which MBD7 recognizes methylated DNA and recruits regulators of active DNA demethylation to prevent DNA methylation spread and transcriptional silencing.

RESULTS

ASI2 Is an Anti-Silencing Factor that Prevents Spread of DNA Methylation

We previously developed a screen for anti-silencing mutants in Arabidopsis that employs wild-type (WT) plants that express a cauliflower mosaic virus 35S promoter-driven sucrose transporter 2 (35S::SUC2) transgene and exhibit a short-root phenotype when grown on medium containing sucrose (Figure 1A) (Lei et al., 2014; Wang et al., 2013). Further, these plants express 35S promoter-driven neomycin phosphotransferase II (NPTII) and hygromycin phosphotransferase II (HPTII) transgenes and thus are resistant to kanamycin and hygromycin. We identified a recessive mutant, asi2-1, from a population of WT plants mutagenized with ethylmethane sulfonate. asi2-1 mutant plants developed long roots on sucrose-containing media due to reduced SUC2 expression (Figures 1A, 1B, and S1A) and were sensitive to hygromycin and kanamycin due to reduced expression of HPTII and NPTII (Figures 1B, 1C, S1A, and S1B). We performed chromatin immunoprecipitation (ChIP) and observed reduced occupancy of RNA polymerase II (Pol II) and enrichment of the repressive chromatin mark H3K9me2 at the 35S promoter in asi2-1 mutant plants, suggesting that reduced RNA levels are caused by decreased Pol II transcription from this promoter (Figures S1C and S1D).

Next, we analyzed DNA methylation of asi2-1 and WT plants by genomic bisulfite sequencing. We found that part of the 35S promoter (region A) is hypermethylated in WT plants, but this hypermethylation extends to the neighboring region (region B) in asi2-1 mutant plants (Figure 1D). Thus, the spread of DNA methylation at the 35S promoter may silence the transgenes in asi2-1 mutant plants. In support of this hypothesis, treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza) suppressed the kanamycin sensitivity of asi2-1 mutant plants (Figure 1C) and restored the expression of NPTII and SUC2 (Figure S1E).

In plants, TEs and other repeats are silenced by DNA methylation and repressive histone modifications (He et al., 2011; Law and Jacobsen, 2010; Tariq and Paszkowski, 2004). We found that the expression of At1G26380, a gene in a repetitive gene cluster (Qian et al., 2012), is reduced in asi2-1 (Figure 1E). Similarly, the expression of the TE At1TE04710 is also decreased in asi2-1 (Figure 1F). Expression of some repeats, like ONSEN and TSI, can be induced by prolonged heat treatment (Ito et al., 2011). We observed reduced heat-induced expression of ONSEN and TSI in asi2-1 compared to WT plants (Figure 1F). In addition, At1G26380, At1TE04710, TSI, and ONSEN display increased DNA methylation in asi2-1 (Figures S1F). Thus, the recessive asi2-1 mutation causes the DNA hypermethylation and silencing of transgenic and some endogenous loci, thereby indicating an anti-silencing role for ASI2.

The Methyl CpG-Binding Protein MBD7 Is Encoded by ASI2

Through map-based cloning, we found that asi2-1 has a C-to-T point mutation in MBD7 (AT5g59800) that creates a premature stop codon (Figure 2A). The long-root phenotype of asi2-1 could be complemented by native promoter-driven MBD7 fused with either a 4xMYC tag or a GFP tag (Figure 2B). RT-PCR assays indicated that the MYC-tagged WT MBD7 is able to rescue the suppressed expression of 35S::SUC2 and 35S::HPTII transgenes in asi2-1 (Figure S2A). Further, we found that MYC-tagged MBD7 protein was enriched at the 35S transgene promoter by ChIP-qPCR (Figure 2C). These results confirm that the transgene silencing in asi2-1 was caused by the mutation in MBD7. We thus refer to the asi2-1 mutant as mbd7-1 hereafter. We also introduced the 35S::SUC2 transgene into a T-DNA insertion allele of MBD7 (SAIL_697_E08/mbd7-2) by crossing the T-DNA mutant with WT plants. As expected, mbd7-2 mutant plants containing the 35S::SUC2 transgene also showed a long-root phenotype (Figure S2B). These results demonstrate that MBD7 dysfunction causes the transgene silencing phenotypes in plants.

The mbd7-1 Mutation Induces DNA Hypermethylation at Endogenous Genomic Regions

Whole-genome bisulfite sequencing identified 1,390 differentially methylated regions (DMRs) in mbd7-1, among which 1,144 are hypermethylated and 246 are hypomethylated. Five hypermethylated cytosines (HMCs) in mbd7-1 were selected for validation by Chop-PCR, an assay in which genomic DNA is digested with a methylation-sensitive restriction endonuclease and then tested as a template for PCR with primers flanking the restriction sites. We observed amplification from genomic digests of mbd7-1 but not WT DNA (Figure 2D), consistent with
hypermethylation of the candidate sites in mbd7-1. The greater number of hypermethylated sites compared to hypomethylated sites in mbd7-1 suggests that MBD7 mainly antagonizes DNA methylation.

We found that about 85% (974) of the hypermethylated DMRs in mbd7-1 are located in TEs, with no obvious preference for any specific type (Figure S2C). The DNA methylation levels are increased at these TE DMRs in CG, CHG, and CHH sequence contexts, and the increase is independent of TE length (Figure S2D).

In mbd7-1 mutant plants, hypermethylated DMRs are concentrated near centromeric and pericentromeric regions (Figure 2E). To test whether MBD7 preferentially regulates TEs located at these regions, we compared the number of hypermethylated DMR-associated TEs to the total number of TEs across consecutive 500-kb windows. This density distribution of hypermethylated TEs across five chromosomes showed no obvious preference for centromeric or pericentromeric TEs, but rather that the concentration of hypermethylated TEs within these regions corresponds to a higher total number of TEs (Figure S2E).

**MBD7 Associates with mCG-Dense Genomic Regions**

MBD7 was previously identified as a methyl CpG-binding protein (Zemach and Grafi, 2007); however, its genomic targets are not known. To identify endogenous MBD7 targets, we performed ChIP using plants expressing native promoter-driven MBD7-4xMYC followed by high-throughput sequencing (ChIP-seq). We identified 2,452 peaks of MBD7 enrichment on chromatin; 1,930 (78.7%) are within TE regions, 94 (3.8%) are within intergenic regions, and 401 (16.4%) correspond to genes (202 of the genes contain annotated repeats). The pattern of chromosomal distribution of MBD7 binding peaks is similar to that of mbd7-1 hyper-DMRs (Figures 3A and 2E), and approximately 63% of the mbd7-1 hyper-DMRs overlap with MBD7 binding peaks.
We examined the correlation between MBD7 binding and DNA methylation in CG, CHG, and CHH contexts. First, the genome was divided into 1-kb regions, and MBD7 enrichment was calculated for each region. The top 1% of MBD7-enriched regions was selected for further analysis. To evaluate the influences of DNA methylation level on MBD7 enrichment, we ranked these regions by CG, CHG, and CHH methylation levels and generated a heat map of MBD7 enrichment. However, no pattern of MBD7 enrichment was evident for any of the three ranks (data not shown), suggesting that the methylation level of individual CG, CHG, or CHH motifs does not determine MBD7 binding.

Methylation density is a value reflecting both methylation level and methyl cytosine density for a region, and it is equal to the sum of methylation percentages of individual cytosines in a region normalized by the region length (Baubec et al., 2013). To consider not only methylation level but also the methyl cytosine density of a region, we calculated the methylation density for 1-kb genomic regions. When these regions were ranked by mCG, mCHG, and mCHH densities, the heat map pattern indicated that MBD7 enrichment is associated with CG methylation density rather than with CHG and CHH methylation densities (Figure 3B), which is consistent with previous in vitro finding that MBD7 can bind to mCG sites but not to mCHG sites (Zemach and Grafi, 2007). The tight relationship between MBD7 enrichment and CG methylation density genome wide (instead of the top 1%) was supported by an analysis using a previously described method (Baubec et al., 2013), where all 1-kb genomic regions were ranked from left to right by their CG methylation density, and their MBD7 enrichment and CG methylation density values are indicated by red and blue lines, respectively (Figure S3A). When the same method was used, no correlation was observed between MBD7 enrichment and methylation density in either the CHG or CHH context (data not shown).
The majority of MBD7 binding sites are in TE regions. We analyzed the CG methylation density of all TE-associated 1-kb windows (TE windows) and all gene-associated 1-kb windows (gene windows) and found that TE windows generally have higher CG methylation density than gene windows (Figure 3C), as expected. To test whether the TE-preferred binding of MBD7 is due to the higher CG methylation density of TE or due to other TE-specific features, we selected TE windows and gene windows with comparable CG methylation density and then compared their MBD7 enrichment (Figure 3D). We found a greater enrichment for MBD7 in TEs than in genes, even when they had comparable mCG densities (Figure 3D); this pattern was consistent for all the ranges of mCG density we tested (Figure S3C). This result suggests that MBD7 may not bind to all regions with a high mCG density, but instead it may recognize and bind to mCGs located in TEs.

### MBD7 Binding Coincides with Its Role in Preventing DNA Hypermethylation

To investigate the correlation of MBD7 binding with its influence on DNA methylation, we calculated MBD7 enrichment and the number of hyper differentially methylated cytosines (DMCs) in the *mbd7-1* mutant for each 2-kb genomic region. Interestingly, the numbers of hyper DMCs in the *mbd7-1* mutant positively correlated with MBD7 enrichment; most of the 2-kb bin genomic regions with MBD7 enrichment (4,474) have higher numbers of hyper DMCs (>50 per 2 kb bin), compared to the numerous 2-kb bin genomic regions without MBD7 enrichment (22,894) (Figure 3E). The result indicates that MBD7 binding to chromatin coincides with its function in preventing DNA hypermethylation.

To further understand the influence of MBD7 on DNA methylation at its binding sites, we compared the methylation levels between the WT and *mbd7-1* at MBD7-binding regions. Two groups of control regions were randomly selected, and their methylation levels were also calculated. In *mbd7-1*, mCHG, mCHH, as well as mCG levels were elevated at MBD7-binding regions but not in control regions (Figure S3C). Boxplot analysis indicated that the increase in average methylation levels at the MBD7-binding regions is due to increases in the majority of the regions rather than extremely large increases in a small number of regions (Figure S3D).

The ChIP-seq data also showed an enrichment of MBD7 protein in the 35S promoter region (Figure 1D), which is consistent with the ChIP-qPCR result (Figure 2C). In addition, the ChIP-seq data revealed MBD7 enrichment at At1G26380, At1TE04710, ONSEN, and TSI regions (Figure S1F), which had increased DNA methylation in *mbd7-1*. These results suggest that physical binding of MBD7 at these loci attenuates the silencing of these loci (Figures 1E and 1F) by preventing DNA hypermethylation.
**idm3 Mutants Phenocopy mbd7 Mutants**

From the same mutant screen, we isolated Increased DNA Methylation 3–1 (idm3-1) and idm3-2, which showed a long-root phenotype on sucrose-containing media and reduced SUC2 and HPTII transcript levels (Figures 4A and 4B). Map-based cloning of idm3-1 and idm3-2 identified missense mutations in At1G20870 (Figure 4C). To further confirm that the mutations in At1G20870 are responsible for the anti-silencing defects in the idm3 mutants, we transformed a native promoter-driven WT At1G20870 gene fused with 4xMYC into the idm3 mutants and found that the root growth as well as SUC2 and HPTII transgene expression phenotypes were rescued (Figures 4A and 4B). IDM3 is predicted to encode an alpha-crystallin domain (ACD) protein localized to the nucleus (Figure S4A) and is similar to IDM2 (Qian et al., 2014) (Figure S4B). Although both IDM2 and IDM3 belong to the family of small heat shock proteins, like IDM2 (Qian et al., 2014), IDM3 expression is not heat inducible (Figure S4C).

Genomic bisulfite sequencing of idm3-1 mutant plants revealed that DNA methylation spread to neighboring sequence in the 35S transgene promoter (Figure 1D). Consistent with the notion that increased DNA methylation was responsible for the silencing of the transgenes, expression of the 35S::SUC2 transgene was restored in idm3 mutants after treatment with the cytosine methylation inhibitor 5-aza, as revealed by RT-qPCR (Figure 4D). The 5-aza treatment also increased the expression of the 35S::SUC2 transgene in the WT (Figure 4D). The result suggests that the transgenes were already subjected to some DNA methylation-dependent silencing in WT plants, which is consistent with the high level of DNA methylation observed within region A of the 35S promoter (Figure 1D).

As in mbd7–1, there were many more hyper-DMRs (1,703) than hypo-DMRs (274) in idm3-1. Approximately 47% (799) of the hyper-DMRs are also hypermethylated in mbd7–1. Chop-PCR analysis confirmed that several endogenous genomic sites were hypermethylated in idm3-1 mutant plants, as in mbd7–1 (Figure 2D). Because we suspected that the idm3-1 and idm3-2 point mutations represent weak alleles, and also because we could not find any T-DNA insertion alleles of idm3, we used the CRISPR/Cas system (Mao et al., 2013) to generate two new alleles, idm3-3 and idm3-4. In idm3-3, a 753-bp fragment was deleted from the IDM3 gene. In idm3-4, a frameshift mutation at the 5’ region of IDM3 created a premature stop changes are normalized to transcript levels in WT. *p < 0.01 compared to WT; NS, not significant compared to WT; **p < 0.01 compared to idm3-1 (two-tailed t test).

(C) A diagram of the IDM3 gene showing the mutation sites in idm3 mutants. Boxes and lines denote exons and introns, respectively.

(D) Transcriptional silencing of 35S::SUC2 in idm3 mutants can be released by chemical inhibition of DNA methylation. Plants were treated with the DNA methylation inhibitor 5-aza or the solvent DMSO as a control. Gene expression levels were normalized to that of DMSO-treated WT plants. RT-qPCR results are means ± SD of three biological replicates, where the fold changes are normalized to transcript levels in WT. p < 0.01 compared to WT

E) Analysis of DNA methylation levels at the DT76 and DT77 loci in idm3-3 and idm3-4 mutants by Chop-PCR.

See also Figure S4.

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**Figure 4. IDM3 Functions in Preventing Transcriptional Silencing**

(A) The idm3 mutants were isolated based on its long root phenotype when grown with exogenous sucrose. Transgenic expression of the WT IDM3 gene rescued idm3 root growth phenotype. The ros1–13 and idm1–9 were used as controls.

(B) IDM3 dysfunction suppresses SUC2 and HPTII transgene expression. RT-qPCR results are means ± SD of three biological replicates where the fold levels were normalized to that of DMSO-treated WT plants. RT-qPCR results are means ± SD of three biological replicates, where the fold changes are normalized to transcript levels in WT. *p < 0.01 compared to WT; NS, not significant compared to WT; **p < 0.01 compared to idm3-1 (two-tailed t test).

(C) A diagram of the IDM3 gene showing the mutation sites in idm3 mutants.

(D) Transcriptional silencing of 35S::SUC2 in idm3 mutants can be released by chemical inhibition of DNA methylation. Plants were treated with the DNA methylation inhibitor 5-aza or the solvent DMSO as a control. Gene expression levels were normalized to that of DMSO-treated WT plants. RT-qPCR results are means ± SD of three biological replicates, where the fold changes are normalized to transcript levels in WT. *p < 0.01 compared to WT; NS, not significant compared to WT; **p < 0.01 compared to idm3-1 (two-tailed t test).

(E) Analysis of DNA methylation levels at the DT76 and DT77 loci in idm3-3 and idm3-4 mutants by Chop-PCR.
MBD7 Interacts with IDM2 and IDM3

In order to understand how MBD7 functions, we identified MBD7-interacting proteins by performing immunoprecipitation (IP) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with native promoter-driven MBD7-4xMYC. WT plants not expressing MBD7-4xMYC served as controls. Of the proteins co-precipitated with anti-MYC antibodies in the MBD7-4xMYC transgenic plants but not in the control plants, four had been uncovered from the 3S:SUC2 transgene-based screen as anti-methylation factors: MBD7 itself, IDM1 (Qian et al., 2012), IDM2 (Qian et al., 2014), and IDM3 (Figure 5A). Similar IP-LC-MS/MS experiments were carried out using idm3-1 mutant plants complemented with native promoter-driven IDM3-4xMYC. In the anti-MYC immunoprecipitates, we identified not only IDM3 itself but also MBD7, IDM2, and IDM1 (Figure 5A). In addition, in anti-GFP immunoprecipitates from idm1-1 mutant plants complemented with native promoter-driven IDM1-1GAFAV-4xHA, we found IDM1 itself and IDM2, IDM3, and MBD7 (Figure 5A). These results show that MBD7 is associated with IDM3, IDM2, and IDM1 in vivo.

Yeast two-hybrid (Y2H) assays were used to determine whether MBD7 interacts directly with IDM1, IDM2, and IDM3. Yeast cells expressing binding domain (BD)-MBD7 and activation domain (AD)-IDM3 were able to grow on both minus-three media (media lacking leucine, tryptophan, and histidine) and minus-four media (media lacking leucine, tryptophan, histidine and adenine) (Figure 5B), suggesting a strong interaction between MBD7 and IDM3. BD-MBD7 and AD-IDM2 co-transfected yeast cells grew on minus-three media but not on minus-four media (Figure 5C), indicating a relatively weaker interaction between MBD7 and IDM2. MBD7 did not interact with IDM1 in the Y2H assay (data not shown). The interaction between MBD7 and IDM3 was confirmed in a split luciferase complementation assay in protoplasts (Figure 5D). The split luciferase complementation assay also indicated an interaction between MBD7 and IDM2, although this interaction is much weaker than that between MBD7 and IDM3, as indicated by the relative luciferase activities (Figure 5D).

The MBD7 interactions with IDM3 and IDM2 were also confirmed using a split luciferase complementation assay in tobacco leaves (Figure 5E). Interestingly, IDM3 also interacted with IDM2 in the assay. We observed an interaction between IDM2 and IDM1 in the assay, which is consistent with previous results (Qian et al., 2014; Zhao et al., 2014). Like IDM2, IDM3 also interacted with IDM1 in the split luciferase complementation assay (Figure 5E). The Y2H and split luciferase complementation results show that MBD7 interacts with IDM3 and IDM2, and that IDM3 interacts with IDM2 and IDM1. These results are consistent with our IP-LC-MS/MS data (Figure 5A) that showed MBD7 association with IDM3, IDM2, and IDM1 in vivo.

MBD7 Affects a Subset of Genomic Regions Targeted for Active DNA Demethylation

MBD7 contains three MBD domains and a C-terminal domain known as the StkC domain (Figure 5B). We performed the Y2H assay with different deletion mutants and found that both IDM2 and IDM3 bind to the StkC domain of MBD7 but not the three MBD domains (Figures 5B and 5C). To test whether StkC, the protein interaction domain, is important for the anti-silencing function of MBD7, we separately transformed the native promoter-driven full-length MBD7 coding sequence (proMBD7: MBD7:4xMYC) and native promoter-driven MBD7 containing only the three MBD domains (proMBD7:3MBD-4xMYC) into mbd7-1. Unlike the full-length MBD7 protein, the three MBD domains could not rescue the root phenotype of mbd7-1 (Figure 5A), indicating that the StkC domain is required for the anti-silencing function of MBD7, at least at the transgene loci.

Our IP-LC-MS/MS results indicated an association between MBD7 and IDM1 in vivo (Figure 5A). We showed previously that IDM1 is a histone H3 acetyltransferase that catalyzes H3K18 and H3K23 acetylation (Qian et al., 2012) and that H3K18ac and H3K23ac marks are reduced at the silenced 3S promoter in idm1 mutant plants (Qian et al., 2014). ChiP-qPCR assays revealed that H3K18ac and H3K23ac levels are reduced in mbd7-1 mutant plants at the 3S promoter, as in idm1-9 mutants (Figures 5B and 5C). The results are consistent with the notion that MBD7 and IDM1 are in the same protein complex in vivo and suggest that MBD7 is important for IDM1 function in plants.
Figure 5. MBD7 Physically Interacts with IDM2 and IDM3

(A) Detection of proteins that associate with MBD7, IDM3, or IDM1. Proteins were detected by LC-MS/MS following IP of MYC-tagged MBD7, MYC-tagged IDM3, or GFP-tagged IDM1. There were two biological replicates for each of the protein purifications; one representative result is shown here.

(B) Tests of IDM3-MBD7 interaction by yeast two-hybrid assays. Full-length protein (MBD7 full) and truncated forms (3MBD and STKC) of MBD7 were fused with the GAL4 binding domain (BD). Full-length IDM3 was fused with the GAL4 activation domain (AD).

(C) Purification of proteins associated with MBD7, IDM3, or IDM1.

(D) Relative LUC activity for different yeast two-hybrid assays.

(legend continued on next page)
the enrichment was substantially reduced in mbd7-1 mutant plants (Figures 6B and S6E). These results suggest that IDM1 binding to target loci is MBD7 dependent.

Our results suggest that MBD7 affects only a specific subset of genomic regions that require IDM1 or IDM2 for DNA demethylation. To understand how MBD7 may achieve this specificity, we analyzed the CG methylation density of the different groups of hyper-DMRs. In idm1, idm2, and idm3 mutants, the hyper-DMRs that overlap with those in mbd7-2 have higher CG methylation density than the non-overlapping hyper-DMRs. Simulations with randomly selected regions do not show a significant difference (Figure S6F). The results suggest that MBD7 functions together with IDM1 and IDM2 preferentially at genomic regions with a high CG methylation density, consistent with the requirement of high mCG density for MBD7 binding. Indeed, analyses of the MBD7 ChIP-seq data showed that MBD7 is more highly enriched at the dense mCG/overlapping hyper-DMRs than at the non-overlapping hyper-DMRs (Figure S6G). No significant difference was found using the control ChIP-seq data from WT plants (Figure S6G).

DISCUSSION

We have found that MBD7 and IDM3 are two cellular anti-silencing factors that inhibit DNA hypermethylation at some genomic regions and prevent transcriptional gene silencing. DNA methylation is a conserved epigenetic mark that silences TEs and other invasive elements (He et al., 2011; Law and Jacobsen, 2010). Many plant and animal genomes have abundant TEs, such that the genes are islands in the sea of TEs (Bennetzen and Wang, 2014). Methylation at TEs may spread to and silence adjacent, transcriptionally active genes. Active DNA demethylation is one mechanism for preventing DNA methylation spread to protect nearly genes. For example, the EPF2 gene, which controls the size of the stomatal stem cell population in leaf epidermis, is close to a methylated TE in Arabidopsis, and active DNA demethylation is required to prevent methylation spreading and transcriptional silencing of EPF2 (Yamamuro et al., 2014). In the DNA demethylase ros1 mutants, EPF2 is silenced by DNA methylation that spreads from the proximal TE, resulting in an over-production of stomatal lineage cells (Yamamuro et al., 2014). Many imprinted genes in plants require the DNA demethylase DME for DNA demethylation and expression in the endosperm, because these genes evolved to have TEs in or near their regulatory sequences (Gehring et al., 2009). Our findings reveal that MBD7 associates with methylated DNA and recruits other anti-silencing factors to create a permissible chromatin environment for binding of DNA demethylases such as ROS1. Thus, our results suggest that MBD7 and the IDM proteins limit the spread of DNA methylation by promoting active DNA demethylation.

Active DNA demethylation is also necessary for pruning the DNA methylation landscape of many TEs and is thus important for preventing over-silencing of TEs (Zhu et al., 2007). In addition, it has been known for a long time that DNA methylation can cause transcriptional silencing of transgenes in plants (Matzke et al., 1989). It is important to understand how transcriptional silencing of transgenes can be avoided or prevented in order to keep transgenic traits stable in the agricultural biotechnology industry.

The key enzymes for active DNA demethylation have been identified in recent years. In vertebrates, active DNA demethylation is initiated by deamination or oxidation of 5mC by AID/APOBECs or TET enzymes, respectively, followed by the DNA glycosylase TDG or MBD4 (Rai et al., 2008; Kohli and Zhang, 2013), whereas in plants, active DNA demethylation is initiated by the ROS1/DME family of 5mC DNA glycosylases (Zhu, 2009). Now the DNA demethylation enzymes are targeted to specific genomic loci is poorly understood. In plants, the histone acetyltransferase IDM1 is required for targeting ROS1 to a subset of genomic regions for demethylation (Qian et al., 2012). Although it is not known how the H3K18 and H3K23 acetylation marks created by IDM1 affect ROS1 targeting, this regulation may be considered an "acetylation switch," analogous to the model proposed for targeting of the chromatin remodeling complex SWR1 in yeast (Ranjan et al., 2013). Like IDM1, IDM2 regulates the demethylation of a similar subset of genomic loci targeted by the ROS1 family of demethylases (Qian et al., 2014). IDM2/ROSS is a nuclear ACD protein that interacts with IDM1 and affects its H3K18 acetylation activity (Qian et al., 2014; Zhao et al., 2014). Interestingly, we have now identified IDM3, an ACD protein that is closely related to IDM2 and is also required to prevent DNA hypermethylation and gene silencing. MBD7 is an MBD protein that contains three MBD domains and a C-terminal Stk domain. Consistent with its ability to bind methylated CpGs in vitro (Zemach and Graft, 2007), our ChIP-seq assays revealed that genome-wide binding of MBD7 correlates with the density of methylated CG but not methylated CHG or CHH sites. Our results suggest that MBD7 does not bind to all regions with a high mCG density, but instead may preferentially recognize and bind to mCGs located in TEs. It is possible that in addition to high mCG density, TE features such as heterochromatic histone marks may also contribute to MBD7 binding. We showed that MBD7 binding is coincident with the role of MBD7 in preventing DNA hypermethylation. Therefore, instead

(C) Tests of IDM2-MBD7 interaction by yeast two-hybrid assays. Full-length protein (MBD7 full) and truncated forms (9MBD and STKC) of MBD7 were fused with BD. Full-length IDM2 was fused with AD.

(D) Analyses of IDM2-MBD7 and IDM3-MBD7 protein interactions by split luciferase complementation assays in Arabidopsis protoplasts. The co-transfected Gus gene was used to standardize protoplast transfection efficiency. Protoplasts expressing the tested protein and proteins unrelated to gene silencing including ABA-hypersensitive germination 1 (AHG1) and PYR1-like protein 13 (PYL13) served as negative controls.

(E) Examination of protein interactions between ROS1, IDM1, IDM2, IDM3, and MBD7 by split luciferase complementation assays in tobacco (N. benthamiana) leaves. Luciferase activities were detected at 48 hr post infiltration. White circles indicate leaf region that were infiltrated with Agrobacterium strains containing the indicated constructs. See also Figure S5.
Figure 6. Shared Hyper-DMRs between mbd7-2 and Other Anti-Silencing Mutants and the Effect of mbd7 on IDM1 Binding to Chromatin

(A) Levels of DNA methylation and MBD7 chromatin enrichment at several shared hyper-DMRs (dashed boxes) in the mbd7-2, idm3-3, idm2-1, idm1-1, and ros1-4 mutants. Total DNA methylation levels of boxed regions in each mutant were quantified from the whole-genome bisulfite sequencing data and shown in the bottom panel.

(B) Effect of mbd7 on IDM1 protein enrichment at the shared hyper-DMRs. ChIP against IDM1-3xHA was performed in WT, IDM1-3xHA/idm1-1, and IDM1-3xHA/mbd7-1. Promoter of ACTIN7 was used as a control region. All error bars indicate SD, n = 3. *p < 0.01; NS, not significant (two-tailed t test).

See also Figure S6.
of reading the DNA methylation signal to cause silencing, MBD7 interprets the DNA methylation signal to avoid DNA methylation spread or hypermethylation to prevent silencing.

MBD7 interacts directly with both IDM2 and IDM3. Like IDM2 (Qian et al., 2014; Zhao et al., 2014), IDM3 also interacts with IDM1. In addition, IDM2 and IDM3 interact with each other. Our IP-LC-MS/MS results show that MBD7 is associated with IDM1 as well as with IDM2 and IDM3 in vivo. The IP-LC-MS/MS results indicated that the amounts of IDM1 and IDM2 that were co-immunoprecipitated with MBD7 or IDM3 were low, compared to the amounts of IDM3 or MBD7 (Figure 5A). The results suggest that while MBD7 and IDM3 may exist in a tight complex, IDM2 and IDM1 are more loosely or transiently associated with the complex. In addition to having three MBD motifs, MBD7 also contains a Stkc domain that is conserved in plant domain proteins. The H3K18ac and H3K23ac marks created by IDM1 then allow ROS1 and related DNA demethylases to be recruited to restrict methylation spread or to prevent hypermethylation by active demethylation.

**Figure 7. A Working Model of the Functions for MBD7 and IDM3 in Anti-Silencing**

MBD7 binds to highly methylated genomic regions through its MBD motifs and uses its Stkc domain to bind to IDM3 and IDM2, thus bringing IDM1 to the methylated DNA. The H3K18ac and H3K23ac marks created by IDM1 then allow ROS1 and related DNA demethylases to be recruited to restrict methylation spread or to prevent hypermethylation by active demethylation.

Nevertheless, the single MBD domain of IDM1 may help anchor IDM1 to the genomic sites once it is recruited by MBD7. Our results suggest that MBD7 and the three IDM proteins form a previously unknown anti-silencing complex that promotes DNA demethylation. Protein complexes that promote DNA methylation and transcriptional gene silencing have been well studied (Matzke and Mosher, 2014). In contrast, nothing has been known in any system about protein complexes that function in regulating DNA demethylation. Our results suggest that MBD7 binds to highly methylated genomic regions through its MBD motifs and uses its Stkc domain to bind to IDM3 and IDM2, thus bringing IDM1 to the methylated DNA (Figure 7). The H3K18ac and H3K23ac marks created by IDM1 then allow ROS1 and related DNA demethylases to be recruited to restrict methylation spread or to prevent hypermethylation by active demethylation (Figure 7). MBD7 may also recruit histone modification enzymes other than IDM1 to help target the DNA demethylases, since IDM1 only affects a subset of genomic regions demethylated by ROS1 and related 5mC DNA glycosylases. It is possible that at some genomic regions MBD7 may affect DNA methylation by preventing the establishment or maintenance of DNA methylation rather than by facilitating active DNA demethylation. On the other hand, not all genomic regions subjected to active DNA demethylation are affected by MBD7, indicating the presence of MBD7-independent mechanisms for targeting the DNA demethylases.

**EXPERIMENTAL PROCEDURES**

**Plant Materials, Mutant Screening, and Map-Based Cloning**

WT in this study refers to transgenic plants expressing the phosphate starvation responsive AtPT2 promoter driven firefly luciferase reporter gene and 35S::NPTII (Karthetakeyan et al., 2002) that were later transformed with a construct containing the CaMV 35S promoter-driven sucrose transporter 2 (35S::SUC2) and 35S::HPTII transgenes (Lei et al., 2011). An EMS-mutagenized pool of plants was generated and screened for mutants with a long-root phenotype (Wang et al., 2013). M2 seedlings were grown vertically on 1% MS plates with 2% sucrose and 1% agar. WT plants exhibit severely suppressed root growth on this medium, and we screened for mutants with long-root phenotype among 7-day-old seedlings. Genetic mapping and gene cloning was performed as described previously (Li et al., 2014). Details of plant materials and gene cloning are provided in Supplemental Experimental Procedures.

**RNA Analysis and Chop-PCR**

RNA analysis and Chop-PCR assays were carried out according to Lei et al. (2014). Detailed procedures are described in the Supplemental Experimental Procedures. Primer information is included in Table S1.

**Whole-Genome Bisulfite Sequencing and Data Analysis**

Fourteen-day-old seedlings were used for extraction of genomic DNA. Bisulfite conversion, library construction, and deep sequencing were performed by the Beijing Genomics Institute (BGI) in Shenzhen, China. DMRs were identified according to Qian et al. (2012) with some modifications, which are detailed in Supplemental Experimental Procedures.

**ChiP Assay**

The ChiP assay was performed as described (Wierzbicki et al., 2008). Dynabeads (Invitrogen, cat# 10003D) were used for pre-clearing and antibody binding. The antibodies were anti-H3K3me2 (Abcam, cat# ab1220), anti-H3K18ac (Abcam, cat# ab11919), anti-H3K23ac (Millipore, cat#07-355), anti-MYC (Millipore, cat#09-724), and anti-Pol II (Abcam, cat#ab5408).
MBD7 ChIP-Seq and Data Analysis

proMBD7: gMBD7-4xMYC transgenic plants were used for ChIP-seq, with WT plants as controls. ChIP was performed accordingly to a previously published protocol (Wierzbiicki et al., 2008). ChIP samples were sent to the Genomics Core Facilities of the Shanghai Center for Plant Stress Biology, SIBS, CAS (Shanghai, China) for library construction and illinuma sequencing.

MBD7 binding Peaks were called with SICER (Zang et al., 2009), which compared the two replicates of MBD7-Myc ChIP samples with the two replicates of WT control. The MBD7 protein enrichments were calculated according to Baubec et al. (2013) as follows: Enrichment = \( \log_2 (\frac{8 \times n_{\text{ChIP}}}{n_{\text{Input}}}) \), where \( n_{\text{ChIP}} \) and \( n_{\text{Input}} \) represent the number of mapped ChIP and input tags in the corresponding 1-kb bin, and \( N_{\text{ChIP}} \) and \( N_{\text{Input}} \) are the sums of all mapped tags. In Figure 3E, the relative enrichment value is calculated as: \( \log_2(\text{MBD7 enrichment value}) \) – \( \log_2(\text{WT enrichment value}) \). Detailed methods are described in Supplemental Experimental Procedures.

Yeast Two-Hybrid Assay

Coding sequences of IDM2, IDM3, MBD7, and truncated MBD7 (3MBD or STK) with stop codon were cloned into pDEST22 or pDEST32. Yeast two-hybrid assays were performed as described (Bai et al., 2013).

IP and LC-MS/MS Analysis

For IP, 5 g of floral tissues for each epitope-tagged transgenic line were used. Dynabeads (Thermo Fisher Scientific) conjugated with MYC antibody (Millipore, cat#05-724) were used for IP. For affinity purification as described in Law et al. (2010), the protein samples were subjected to LC-MS/MS analysis as described in Supplemental Experimental Procedures.

Split Luciferase Complementation Assays

Split luciferase complementation assays were performed in tobacco leaves and Arabidopsis protoplasts, the details of which are provided in Supplemental Experimental Procedures.

ACCESSION NUMBERS

All high-throughput sequencing data generated in this study were submitted to NCBI’s Gene Expression Omnibus (GSE58789, GSE58787, and GSE59712). Additional data sets used in this study are GSE33071 (Qian et al., 2012) and GSE49421 (Qian et al., 2014).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at https://dx.doi.org/10.1016/j.molcel.2015.01.009.

AUTHOR CONTRIBUTIONS

J.-K.Z., Z.L., and M.L. designed the study; interpreted the data; and wrote the manuscript. Z.L. and M.L. performed much of the experimental work. X.W., H.Z., D.M., S.M., W.L., W.N., G.M., J.Y., and C.-G.D. contributed to the experiments and discussion of results. K.T. and Z.L. did the bioinformatics analysis. C.H. and W.T. performed the mass spectrometry experiments. C.W. and Z.G. contributed to discussion and interpretation of results.

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REFERENCES


