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De novo DNA methylation activity of METHYLTRANSFERASE 1 (MET1) partially restores body methylation in *Arabidopsis thaliana*

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SUMMARY

Arabidopsis METHYLTRANSFERASE 1 (MET1) controls faithful maintenance of cytosine methylation at CG sites in repetitive regions and central body regions of active genes. If MET1 is removed in a mutant background, CG methylation is lost and is only restored in specific heterochromatic regions that have maintained competence for re-methylation due to the presence of small RNAs and the RNA-directed DNA methylation pathway that controls *de novo* DNA methylation functions. We analysed re-methylation at a locus that loses body methylation in an *met1* mutant. We found that body methylation at this locus is at least partially restored when MET1 is re-introduced into the *met1* mutant background, either via genetic cross or DNA transfer. Re-methylation is region-specific but random with respect to individual CG targets, does not require passage through the germline, and its efficiency appears to be influenced by transcription. This suggests that, at least at some loci, MET1 has *de novo* methylation activity that can restore lost body methylation patterns. We propose that this activity helps to stabilize body methylation patterns, and the random target site selection probably also enhances the variability of body methylation patterns.

Keywords: DNA methylation, body methylation, MET1, CG methylation, *Arabidopsis thaliana*.

INTRODUCTION

DNA methylation in plants targets cytosine residues in all sequence contexts (CG, CNG and CNN), and is controlled by three DNA methyltransferase pathways. CG methylation patterns are faithfully maintained by METHYLTRANSFERASE 1 (MET1), a homologue of the mammalian maintenance methyltransferase Dnmt1 (Finnegan *et al.*, 1996; Kankel *et al.*, 2003). DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), a homologue of mammalian Dnmt3 *de novo* methyltransferases, is responsible for *de novo* methylation of cytosines in all sequence contexts, which are targeted by 24 nt siRNAs generated by the RNA-directed DNA methylation (RdDM) pathway (Xie *et al.*, 2004). CNG methylation is predominantly controlled by the plant-specific CHROMOMETHYLASE 3 (CMT3) (Lindroth *et al.*, 2001), which contains a chromodomain that to binds H3 Lys9 dimethylation (H3K9me2) marks established by histone methyltransferases, predominantly KRYPTONITE/SUVH4 (KYP).

Prominent DNA methylation targets in Arabidopsis are transposable elements and pericentromeric heterochromatin that consists of repetitive sequences and transposable elements (Lister *et al.*, 2008). Transposons and repeats contain methylated cytosines at CG and non-CG contexts, accompanied by enhanced levels of H3K9me2 marks (Bernatavichute *et al.*, 2008) and siRNAs (Cokus *et al.*, 2008; Lister *et al.*, 2008). At least 20% of expressed genes contain CG methylation patterns in central regions excluding promoter and 3′ regions (Zhang and Jacobsen, 2006; Vaughn *et al.*, 2007; Zilberman *et al.*, 2007; Cokus *et al.*, 2008; Reinders *et al.*, 2008; Zhang *et al.*, 2008). In contrast to repeats and transposable elements, these body methylation regions are not enriched in H3K9me2 marks, due to the activity of INCREASE IN BONSAI METHYLATION 1 (IBM1), a jumonji domain-containing histone demethylase that removes H3K9me2 marks from active genes. In an *ibm1* mutant, a large number of genes accumulated H3K9me2 and CNG marks, which reflects the interplay between KYP and CMT3 (Inagaki et al., 2010). A similar increase in CNG and other non CG marks is detected when transcription is interrupted, which led to a model suggesting that IBM may be selectively recruited to transcribed genes (Inagaki *et al.*, 2010).

Heterochromatic DNA methylation harnesses the activity of transposable elements (Lisch, 2009) and contributes to

chromosome stability (Soppe *et al.*, 2002). The significance of body methylation is less well defined. Body methylation is more likely to occur at genes that are longer than average and have more exons, and the affected genes show a slow evolutionary rate, which is in line with a functional importance that does not tolerate mutations very well (Takuno and Gaut, 2011). Body methylation may play an active role in selecting splice regions, a hypothesis that is supported by the predominant presence of CG methylation in exons (Takuno and Gaut, 2011). Alternatively, it has been proposed that body methylation inhibits transcription from cryptic promoters in central gene regions (Zilberman *et al.*, 2007). However, it is difficult to differentiate between cause and consequences, and body methylation may simply be the consequence of transcription (Teixeira and Colot, 2009). A better understanding how body methylation marks are established and maintained may be helpful to understand its significance.

Heterochromatic methylation marks are stable and conserved among ecotypes, while body methylation patterns are polymorphic and highly variable among ecotypes (Vaughn *et al.*, 2007). It has been proposed that the instability of body methylation marks reflects the lack of siRNA signals that are associated with transposable elements and restore faulty maintenance functions of MET1. MET1 controls both heterochromatin and body methylation patterns, and its elimination causes loss of heterochromatic DNA methylation (Finnegan *et al.*, 1996; Saze *et al.*, 2003) and H3K9 methylation (Tariq *et al.*, 2003), and dispersion of pericentromeric sequences away from chromocenters (Soppe *et al.*, 2002). Body methylation regions lost CG methylation in a *met1* mutant and frequently gained CNG methylation (Lister *et al.*, 2008). It has been proposed that in a met1 mutant, IBM1 is recruited to transposons and therefore depleted at body methylation regions, which induces a gain in CNG methylation (Inagaki *et al.*, 2010).

Once CG methylation has been eliminated, its re-establishment is slow or does not occur at all (Vongs *et al.*, 1993; Finnegan *et al.*, 1996; Kankel *et al.*, 2003). One exception to the rule that lost DNA methylation in plants is not efficiently restored is the successful re-methylation of certain repeats over successive generations, based on the guiding role of homologous siRNAs that regulate the presence of CNN methylation marks at these re-methylatable repeats (Teixeira *et al.*, 2009).

Body methylation regions lack siRNAs that could serve as signals for re-establishment of lost methylation marks, in accordance with the high level of methylation variance observed for body methylation regions (Vaughn *et al.*, 2007). To examine the role of MET1 in restoring body methylation in a hypomethylated region, we selected a body methylation locus that does not gain CNG methylation marks in the *met1* mutant. We re-introduced MET1 activity into the *met1* mutant either via genetic crosses or via transfer of an *MET1* transgene. Surprisingly, we find that MET1 restores body methylation, which is region-specific but random with respect to the affected CG sites, and is moderately although not decisively influenced by transcription.

RESULTS

Selection of a model locus to study body methylation

The aim of our study was to determine whether body methylation patterns, once they have been passively lost in an *met1* mutant, could be restored by a re-introduced MET1 function. For this purpose, we selected a model locus, At5g10540 (Figure 1a), which contains a high level of CG body methylation marks in wild-type Columbia, and which loses all body methylation marks in the *met1* mutant without establishing significant levels of novel non-CG methylation marks. To test the influence of transcript levels on body methylation, we selected a T-DNA insertion line, SALK_051859, that shows a significant reduction in At5g10540 transcript levels (Figure 1b). We refer to the T-DNA allele of the At5g10540 body methylation model locus as '*bm*' (body methylation) and to the wild-type At5g10540 allele with full transcriptional activity as '*BM*'.

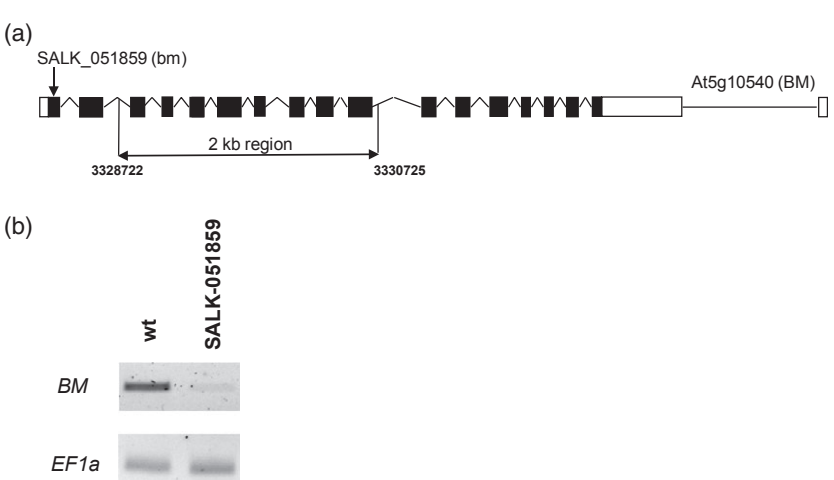

Figure 1. Structure and expression of At5g10540 in wild-type and in the T-DNA insertion line SALK_051859.
(a) Map of At5g10540 (*BM*) with exons marked as boxes and coding regions marked as filled boxes, indicating the location of the 2 kb region (position 3 328 722–3 330 725 on chromosome 5) analysed by bisulfite sequencing and the T-DNA insertion in the first exon in T-DNA line SALK_051859.
(b) RT-PCR analysis of transcript levels of At5g10540 (*BM*), which are significantly reduced in T-DNA insertion line SALK_051859. The low background levels detectable in the knockout line may reflect random transcript initiation or read-through transcription from T-DNA promoters, which is frequently observed in T-DNA lines (Zubko *et al.*, 2011).

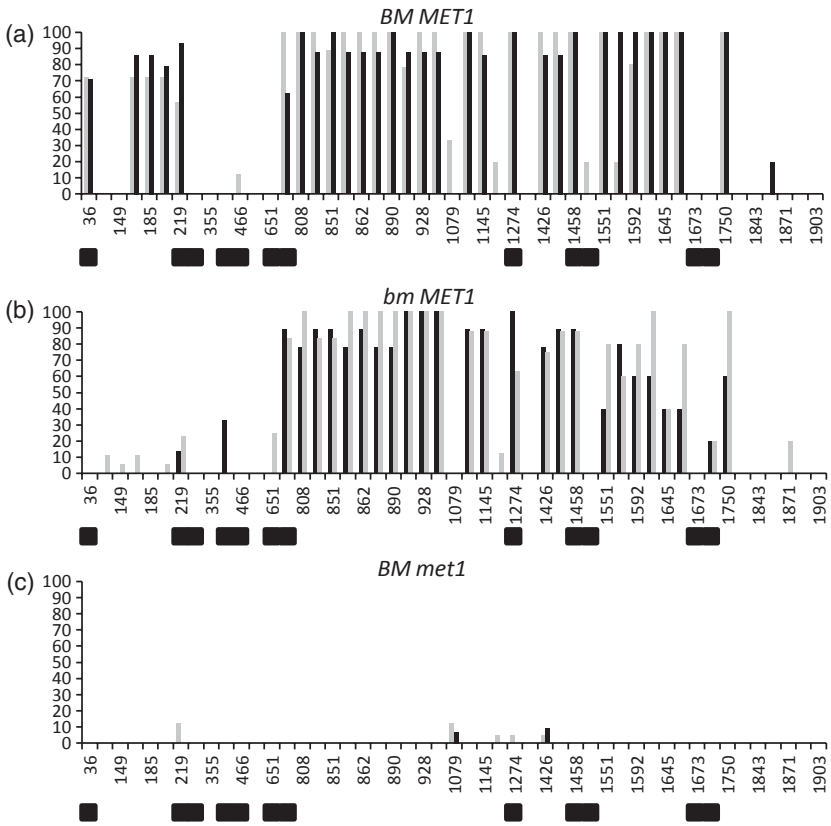

Figure 2. Methylation frequency of At5g10540 at 49 CG sites within the 2 kb region in (a) wild-type Columbia, (b) the *bm* mutant line SALK_051859, and (c) the *met1* mutant.
Numbers refer to the position of a CG target with respect to the start of the analysed region. Grey and black columns represent bisulfite sequencing data for two samples from identical genotypes. Black squares indicate CG positions located within introns.

We characterized CG methylation patterns within a 2 kb region of At5g10540 for the *BM* and *bm* alleles (Figure 2a,b). Overall, CG methylation levels are slightly reduced in the T-DNA line (39.8/42.5%, see Table S1 where two samples were analysed for each genotype) compared to wild-type (54.4/55.1%, again see Table S1), especially in the first third of the 2 kb region. This may indicate a moderate influence of transcription on the efficiency of body methylation. For both the wild-type and the T-DNA line, we examined seedling populations from two plants, and found that comparable regions were methylated in each of the replica lines but that methylation levels for individual CG targets varied, indicating a high level of variability for At5g10540 body methylation. In both the *BM* and *bm* allele, CG methylation is less prominent in introns, but some CGs located within introns are highly methylated, as illustrated by the CGs at positions 1274 and 1458 (Figure 2a,b).

Removal and restoration of At5g10540 body methylation

In the *met1* mutant, CG methylation levels fall to 0.3%/0.8% (Figure 2c), which is equivalent to complete loss of DNA methylation given the fidelity of the bisulfite sequencing technique. DNA methylation levels at non-CG targets did not change significantly in the *met1* mutant compared to wild-type (Table S1). The expression level of At5g10540 did not differ between wild-type and the *met1* mutant (Figure S1).

We used the T-DNA insertion as a tag to distinguish between the *bm* and *BM* alleles in genetic crosses, which allowed us to follow the passage of both alleles from an *met1* mutant background back to a wild-type background with functional MET activity. To analyse re-methylation of a *BM* allele, we crossed a *met1* mutant (*BM/met1*) with the T-DNA insertion line (*bm/MET1*), and selfed two progeny plants (A and B) from which we isolated two F_2 lines each (A1/A2 and B1/B2, respectively) with *BM/MET1* genotypes (Figure S2). Seedlings from each of the four F_2 lines and from four F_3 progeny plants were pooled for bisulfite analysis (Figure 3). We detected re-establishment of CG methylation in all four lines, especially in the central and 3′ section of the 2 kb region. As already observed in wild-type, intron-specific CGs are not exempt from methylation but are under-represented. Lines A and B share similar methylation regions but the individual CG methylation patterns are variable. Even within the two A lines and within the two B lines, methylation targets vary slightly. Overall methylation levels were higher in B lines (24.2%/25.4%, see Table S2) than in A lines (9.6%/14.5%, see Table S2), and methylation frequencies and targets do not differ significantly among F_2 and F_3 generation plants. Re-methylation therefore appears to be region-specific but variable with respect to the selection and methylation intensity of individual CG residues, and, although reasonably conserved, methylation patters also show some variation in the next generation.

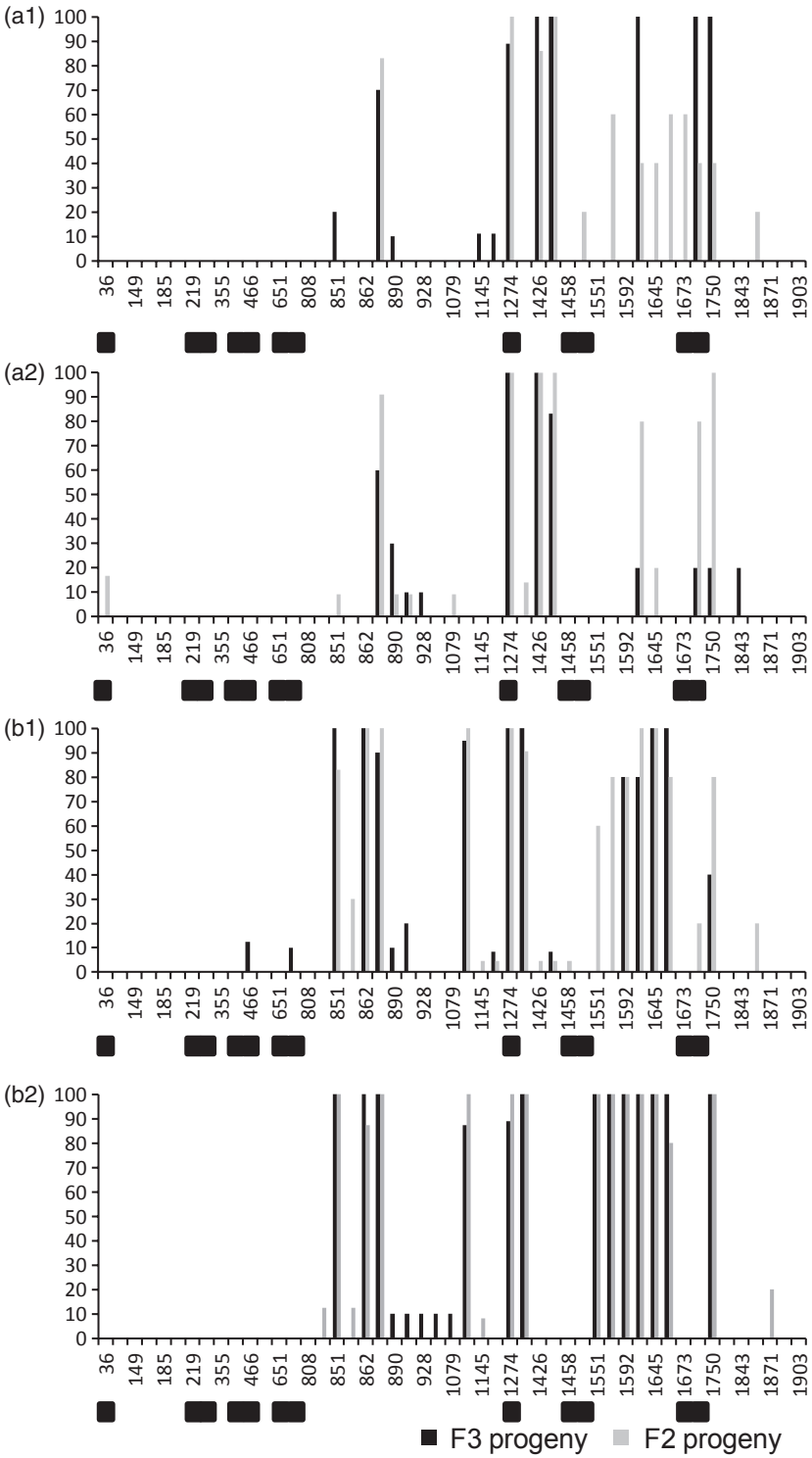

Figure 3. Restoration of methylation patterns at the *BM* allele in F_2 (black) and F_3 (grey) progeny plants derived from two selfed F_1 lines (A and B). For each selfed F_1 plant progeny, two F_2 lines (A1/A2 and B1/B2) were analysed (see Figure S2 for details of crosses).

To test the role of transcription in re-initiation of body methylation, we transferred the *bm* allele into an *met1* mutant background and crossed this line to a wild-type plant. From the selfed progeny of this cross, we selected three lines with *bm/MET1* genotype. As our previous experiments had demonstrated a higher level of diversity among methylation initiation events compared to their propagation, we concentrated on three independent re-methylation events. In all three lines, the *bm* allele becomes methylated at overall levels between 7 and 12.8%, which are slightly lower than the 9.6–25.4% methylation frequency observed for the *BM* allele (Table S2). In accordance with previous

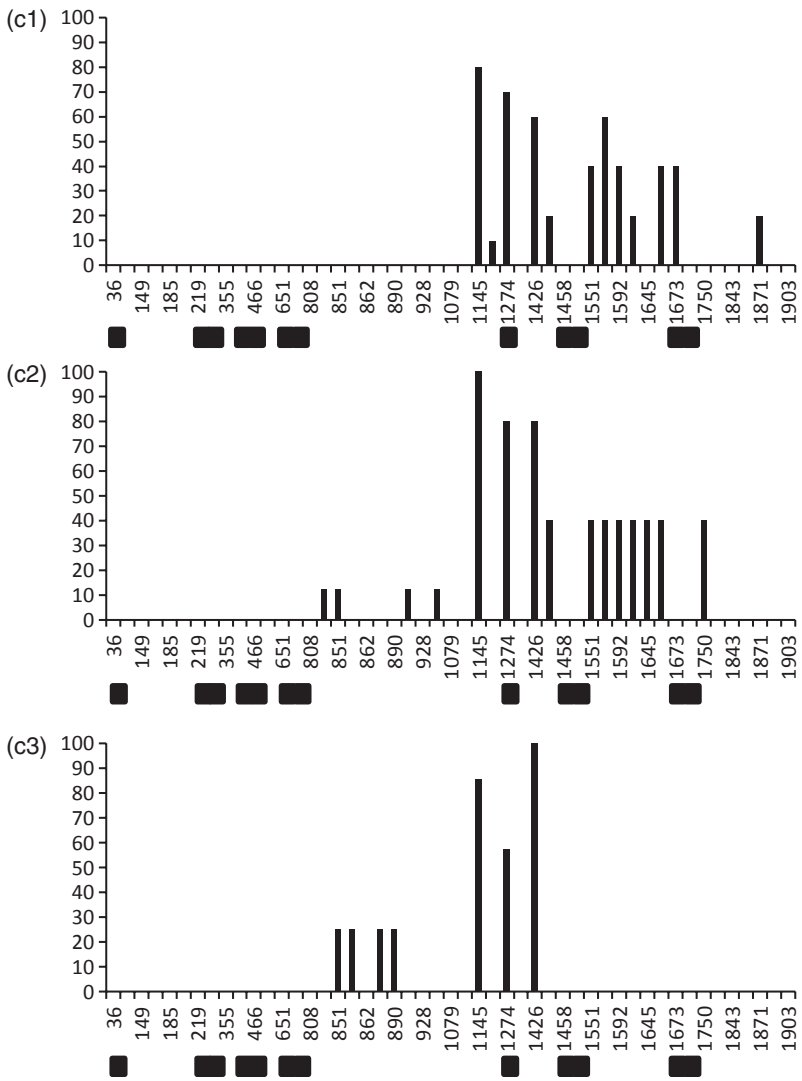

Figure 4. Restoration of methylation patterns at the *bm* allele for three F_2 progeny plants C1-C3 as shown in figure S2 (see Figure S2 for details of crosses).

results, the central region of the 2 kb fragment was a common target in all lines, but methylation of individual CG sites was variable (Figure 4). The dramatic changes in transcript levels between the *BM* and *bm* alleles may therefore have a moderate affect on the intensity of re-methylation, while target selection and variability of methylation patterns are comparable in both alleles.

Re-methylation in *met1* transformants

Genetic crosses of *met1* and wild-type lines not only expose the unmethylated alleles to a functional MET1 protein, they also introduce a methylated homologous allele. This may allow *in trans* interactions between the methylated and the unmethylated allele. To test whether such paramutation-like effects are involved in re-methylation of the *BM* allele, we transformed *met1* with an *MET1* cDNA transgene under the control of the *MET1* promoter. We used a root transformation technology, because we also wanted to examine whether re-methylation required a passage through the germline. We selected three transformants Tr1–3 with different expression levels (Figure 5a). Tr2, the line with the lowest expression levels, did not display significant re-methylation, but lines Tr1 and Tr3 showed re-methylation that was most pronounced in the central region (Figure 5b). In two progeny lines of Tr1, Tr1T1a and Tr1T1b, we detect low levels of CG methylation, with a high variation at individual CG targets. These data imply that re-methylation can occur in the absence of a homologous methylated allele and without passage through the germline. Overall, re-methylation frequencies are lower in transformants than in lines obtained by genetic crossing.

DISCUSSION

MET1 (Finnegan and Dennis, 1993) and its closely related mammalian homologue Dnmt1 (Bestor *et al.*, 1988) have a DNA maintenance function that preserves CG methylation patterns during cell division (Goll and Bestor, 2005), providing

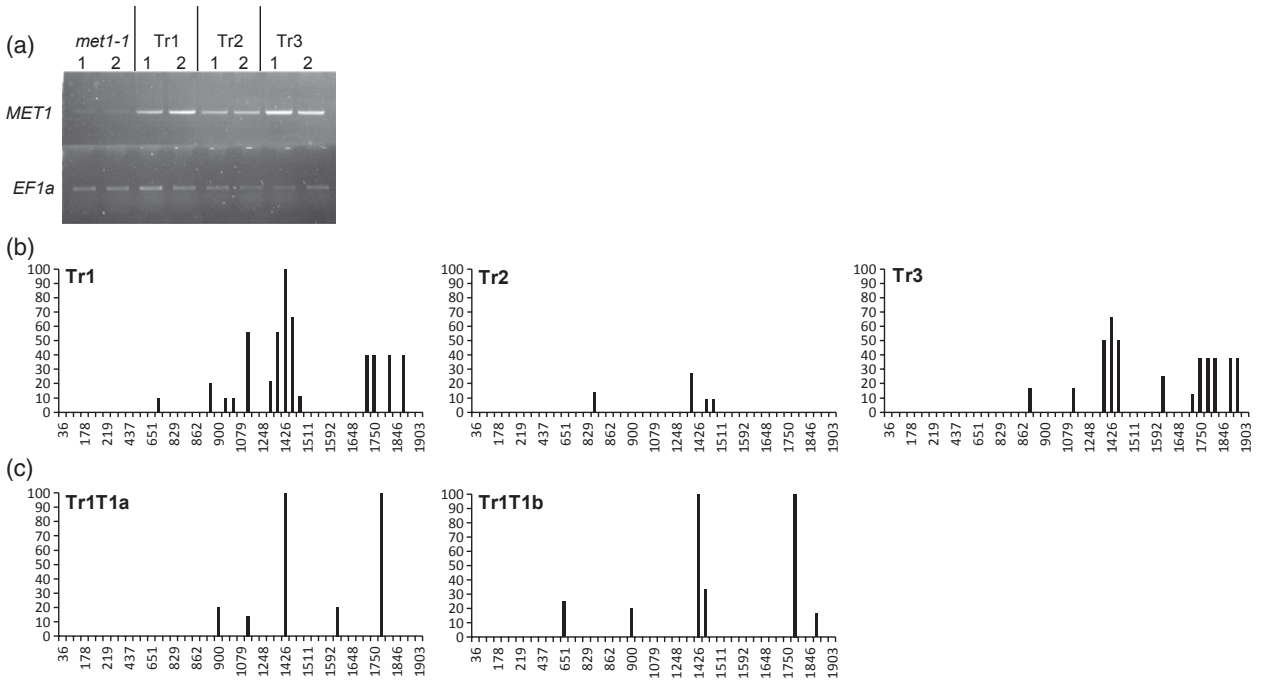

Figure 5. Expression of *MET1* cDNA in the *met1* mutant.
(a) RT-PCR analysis of *MET1* transcript levels in the *met1* mutant and in duplicate samples of three transformants (Tr1–3) expressing an *MET1* transgene. The *met1* transcript is inactive due to a point mutation. *EF1a* expression was measured as a loading control.
(b) Restoration of methylation patterns at the *BM* allele in primary transformants Tr1–3.
(c) Methylation pattern in two progeny plants of Tr1.

heritability to genomic methylation patterns. Maintenance methylation of Dnmt1 activity is based on its strong preference for hemi-methylated DNA (Stein *et al.*, 1982; Yoder *et al.*, 1997), while its CXXC domain specifically binds unmethylated CG dinucleotides, rendering the enzyme inactive as part of an auto-inhibitory mechanism preventing *de novo* methylation (Song *et al.*, 2011). A similar maintenance function for CG dinucleotides has been suggested for MET1, mainly based on genetic experiments that showed specific loss of CG methylation in *met1* mutants and poor re-establishment of methylation after re-introduction of MET1 (Vongs *et al.*, 1993; Finnegan *et al.*, 1996; Kankel *et al.*, 2003).

De novo methylation is established by DRM2 as part of the RdDM pathway (Xie *et al.*, 2004), and MET1 is thought to maintain CG methylation marks once they have been established. However, at certain sites, the RdDM pathway is unable to establish full CG methylation in *met1* mutants, which led to the suggestion that, in addition to its maintenance function, MET1 can act as a site-specific *de novo* methylation enzyme as part of the RdDM pathway (Aufsatz *et al.*, 2004). Our data also argue in favour of a site-specific *de novo* methylation activity of MET1 in restoring body methylation at the analysed locus.

In all lines methylation is re-established within the same region of the BM allele but shows a high level of variability with respect to the methylation efficiency and selection of individual CG targets. Variability is still detectable to a lesser extent when body methylation patterns are transmitted to the next generation. This indicates that MET1-mediated *de novo* methylation is target-specific but variable, and that MET1-mediated maintenance of body methylation patterns has higher fidelity than initiation of body methylation. Introns are less prominent targets than exons, but placement of a CG within an intron does not exclude its methylation, as individual intron CGs can become highly methylated.

It remains unclear how MET1 is attracted to its body methylation target. It has been suggested that CNG methylation may trigger the CG methylation, as the genic methylation level at CG sites positively correlates with CNG methylation for some genes (Inagaki *et al.*, 2010). However, at least for the At5g10540 alleles, there is no evidence for a role of CNG methylation in guiding MET1 re-methylation activity, as CNG levels are very low in wild-type and mutant lines (Table S1). It is also conceivable that MET1 collaborates with other DNA methyltransferases, for example by propagating DRM2-specific *de novo* methylation. However, as we did not observe any significant DRM2-derived methylation in the *met1* mutant, such a model is only likely if MET1 is able to enhance DRM2 activity in a co-operative manner, as has been observed for the repetitive DNA sequence (RPS) transgene (Singh *et al.*, 2008).

It has been proposed that moderately transcribed genes are most likely to be methylated, while genes with very high or low expression levels are less likely to be methylated (Zhang *et al.*, 2006; Zilberman *et al.*, 2007). Our data support this model, as the re-methylation efficiency is higher in the moderately transcribed *BM* allele compared to the low-expression *bm* allele. Transcription may therefore play a role in determining the efficiency of MET1 targeting; however, it does not alter the selection of the central region as the most prominent methylation region, nor does it influence the general variability in selection of individual target sites. It has been proposed that aberrant transcript formation at cryptic promoters within central genic regions favours body methylation (Zilberman *et al.*, 2007). If this is the case, it is unlikely that the level of transcription plays a decisive role in activation of cryptic promoters, as even the significant reduction in transcript levels in the *bm* allele still induces re-methylation. Any transcript-mediated recruitment of a re-methylation function is unlikely to be mediated by the RNA-directed DNA methylation (RdDM) pathway, which regulates re-methylation of transposable elements (Teixeira *et al.*, 2009), given that body methylation is independent of the known RdDM components (Miura *et al.*, 2009). Chromatin signatures of combined transcription-induced histone marks (Roudier *et al.*, 2011) may be alternative targets to which MET1 is guided, either directly or in co-operation with interacting factors. A *de novo* methylation activity for the mammalian MET1 homologue has been reported for the mammalian MET1 homologue Dnmt1, and it was suggested that regulatory factors that interact with Dnmt1 or secondary DNA structures may play a role in targeting Dnmt1 to specific regions (Yoder *et al.*, 1997).

Re-methylation also occurs in transgenic *met1* lines expressing *MET1* cDNA. As observed for re-methylation events in plants derived from genetic crosses, passage through the germline does not significantly increase body methylation rates. Overall, re-methylation efficiencies in *met1* transformants are lower than in lines crossed with wild-type plants. This may reflect differences in MET1 protein levels or may be due to the fact that, in progeny from genetic crosses, one genome carries the normal level of CG methylation so that only half the sites need to be re-methylated, whereas all CG methylation is lacking in the transgenic situation, implying that a great overall level of METI activity is required to restore genome-wide methylation. Finally, it may be due to an enhancing paramutation effect, for example via physical interaction and exchange of epigenetic marks (Stam, 2009) with the methylated wild-type allele, that is co-transferred with *MET1* in genetic crosses. While paramutation-like effects cannot be excluded, re-methylation is clearly possible in the absence of a homologous methylated allele.

We need to be careful not to extrapolate too much from the study of one model gene, as the control mechanism for body methylation may differ for targets depending on the involvement of CNG methylation or other systems that contribute to the methylation of individual loci with various efficiencies. However, our studies do enable us to draw some conclusions regarding the role of MET1 beyond its classical role as a DNA maintenance enzyme. At least for the analysed model locus, we detected a region-specific *de novo* methylation function of MET1 that, due to its variation and the associated moderate maintenance efficiency, may play a decisive role in generating random variability in body methylation patterns. In contrast to its role in faithful propagation of CG methylation patterns, MET1 may therefore have developed an additional function stimulating methylation diversity at body methylation targets.

EXPERIMENTAL PROCEDURES

Plant material and genotyping of mutants

The *ddm2-1/met1-1* (At5g49160) mutant in the Columbia ecotype background was a kind gift from Dr Mittelsten Scheid (Gregor Mendel Institute of Molecular Plant Biology GmbH, Vienna, Austria). The SALK_051859 T-DNA insertion line in the Columbia ecotype background was obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info). All plants were grown in a growth chamber under short-day conditions (8 h light/16 h dark, temperature 22°C, humidity 60%) unless stated otherwise. Genomic DNA for genotyping was extracted from 3–4-week-old leaf tissue as described by Vejlupkova and Fowler (2003). PCR reactions were performed using GoTaq master mix (Promega, http://www.promega.com) according to the manufacturer's instructions.

Genetic crosses

The homozygous SALK_051859 T-DNA insertion line (named *bm*–/–) was crossed with the *met1-1* mutant. The F_1 generation was checked for heterozygosity of both genes (*BM*+/*bm*– *MET1*+/*met1-1*–) and selfed. F_2 progeny plants with the *BM*+/+ *MET1*+/+ genotype were selected and selfed. From the same cross, a line with the *bm*–/*met1-1*–/– genotype was selected and crossed with wild-type Columbia. The F_1 generation was checked for heterozygosity of both genes (*BM*+/*bm*– *MET1*+/*met1-1*–) and selfed. F_2 progeny plants with the *bm*–/– *MET1*+/+ genotype were selected and selfed.

Construct design and root transformation

A construct containing *MET1* cDNA under the control of the *MET1* promoter (*MET1*promoter–*MET1*–*nos*) was generated in two steps. The full-length *MET1* cDNA was cloned into the pGEM-T Easy vector (Promega) and the *MET1* cDNA was subsequently re-cloned into pGreen0179 (http://www.pgreen.ac.uk/JIT/pG0179.htm) and placed under control of the *MET1* promoter (see Appendix S1). Roots were isolated from 3–4-week-old seedlings and used for co-cultivation with *Agrobacterium* as described previously (Valvekens *et al.*, 1988).

Expression and DNA methylation analysis

Total RNA was extracted as described previously (Stam *et al.*, 2000). RNA was treated with DNase (Ambion, http://www.invitrogen.com/site/us/en/home/brands/ambion.html) and cDNA synthesis was performed on 2 μg RNA using Superscript II reverse transcriptase (Invitrogen, http://www.invitrogen.com/) and oligo(dT) primer according to the manufacturer's instructions.

Genomic DNA was isolated (Dellaporta *et al.*, 1983) and subjected to bisulfite treatment using an Epitect bisulfite kit (Qiagen, http://www/qiagen.com) according to the manufacturer's instructions. Five fragments (A, 274 bp; B, 478 bp; C, 439 bp; D, 419 bp; E, 532 bp) were amplified to analyze the methylation pattern of a 2004 bp genomic region of the At5g10540 gene (chromosome 5, 3 328 722–3 330 725 bp). For each line, 6–10 clones were sequenced and sequences were exported into the BioEdit program (Hall, 1999). Aligned sequences were saved in FASTA format and analyzed by the CyMATE program (Hetzl *et al.*, 2007). Each bar on the graph represents the mean methylation level for each CG site. Overall CG methylation levels represent the mean CG methylation level at all analysed sites, and were calculated as the sum of all methylated CGs divided by the number of CG sites in the analysed region. All primers used in this study are listed in Table S3.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The expression level of At5g10540 does not differ between wild-type and the *met1-1* mutant.

Figure S2. Scheme of genetic crosses.

Table S1. Methylation frequency for different target types in wild-type, the *met1* mutant and SALK_051859.

Table S2. Methylation frequencies in *met* mutant lines after restoration of MET1 activity.

Table S3. List of primers used in this study.

Appendix S1. Experimental procedures for genotyping, cloning, root transformation, expression analysis and bisulfite sequencing.

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