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Transduction of RNA-directed DNA methylation signals to repressive histone marks in *Arabidopsis thaliana*

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RNA-directed modification of histones is essential for the maintenance of heterochromatin in higher eukaryotes. In plants, cytosine methylation is an additional factor regulating inactive chromatin, but the mechanisms regulating the coexistence of cytosine methylation and repressive histone modification remain obscure. In this study, we analysed the mechanism of gene silencing mediated by *MORPHEUS' MOLECULE1* (*MOM1*) of *Arabidopsis thaliana*. Transcript profiling revealed that the majority of up-regulated loci in *mom1* carry sequences related to transposons and homologous to the 24-nt siRNAs accumulated in wild-type plants that are the hallmarks of RNA-directed DNA methylation (RdDM). Analysis of a single-copy gene, *SUPPRESSOR OF drm1 drm2 cmt3* (*SDC*), revealed that *mom1* activates *SDC* with concomitant reduction of di-methylated histone H3 lysine 9 (H3K9me2) at the tandem repeats in the promoter region without changes in siRNA accumulation and cytosine methylation. The reduction of H3K9me2 is not observed in regions flanking the tandem repeats. The results suggest that MOM1 transduces RdDM signals to repressive histone modification in the core region of RdDM.

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Introduction

Heterochromatin is an inert structure in the nucleus composed of remnants of transposons and repetitive elements, and rich in repressive histone modifications (Grewal and Elgin, 2007). In fission yeast, repressive histone marks are recognized by a variety of protein complexes, including an RNA-induced transcriptional gene silencing complex and the Snf2/Hdac-containing repression complex that reinforces heterochromatin silencing (Grewal and Elgin, 2007; Cam *et al*, 2009). In mammals, the Mi-2/nucleosome remodelling and deacetylating complexes function to repress transcription through histone deacetylation and binding to methylated cytosines (Bowen *et al*, 2004). In *Arabidopsis thaliana* accession Columbia, the major heterochromatin regions are located around centromeres, nucleolar organizer regions, and a region on the short arm of chromosome 4 called the heterochromatin knob (Fransz *et al*, 2003). Plant heterochromatin is abundant in di-methylated histone H3 lysine 9 (H3K9me2) and cytosine methylation, and maintenance of cytosine methylation at CG contexts is essential for the structural integrity of heterochromatin (Mittelsten Scheid *et al*, 2002; Probst *et al*, 2003). Components of RNA-directed DNA methylation (RdDM) are also required for cytosine methylation, and transposons and repetitive sequences in heterochromatin are maintained in the inactive state through RdDM (Matzke and Birchler, 2005; Slotkin and Martienssen, 2007). Heterochromatin transcripts are processed into 24-nt siRNAs by the action of RNA processing machineries including RNA-DEPENDENT RNA POLYMERASE2 (RDR2) and DICER-LIKE3 (DCL3), and the resulting siRNAs are incorporated into ARGONAUTE4 (AGO4), which directs DNA methylation in regions homologous to the siRNAs by DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) in cooperation with DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1 at all cytosine contexts (CG, CHG, CHH; H = A, T, or C; Matzke and Birchler, 2005; Vaucheret, 2006). Methylated CG and CHG co-localize with H3K9me2, and CHG methylation especially is directly connected to H3K9me2 by CHROMOMETHYLASE3 (CMT3) and KRYPTONITE (KYP) (Jackson *et al*, 2002; Tariq *et al*, 2003; Johnson *et al*, 2007). However, the effect of *drm2* and *cmt3* mutations on symmetric and asymmetric cytosine methylation varies at different loci, and therefore complex, redundant, and locus-specific pathways have been proposed to silence various loci in the genome (Cao and Jacobsen, 2002; Chan *et al*, 2006; Henderson and Jacobsen, 2008).

MORPHEUS' MOLECULE1 (*MOM1*) was identified in a screen for mutants that release transcriptional gene silencing (TGS) of a cluster of transgenes (Amedeo *et al*, 2000). In addition to transgene TGS, *mom1* mutants release silencing of remnants of transposons (Steimer *et al*, 2000; Habu *et al*, 2006) and silent copies of *5S rRNA* genes (Vaillant *et al*,

2006). *mom1* mutants have no effect on global cytosine methylation in the genome and in the target genes (Amedeo *et al*, 2000; Habu *et al*, 2006; Vaillant *et al*, 2006), and maintain morphologically normal chromocenters—nuclear foci consisting of centromeres and other heterochromatin regions (Mittelsten Scheid *et al*, 2002; Probst *et al*, 2003). This contrasts sharply with effects observed in other mutants in which silent genes and transposons are activated with global loss of DNA methylation and alteration of nuclear morphology (Fransz *et al*, 2003). The predicted MOM1 protein has a domain with limited similarity to the helicase domain of SWI2/SNF2 chromatin remodelling proteins (Amedeo *et al*, 2000), but this domain has recently been shown to be dispensable for silencing. Other than the nuclear localization signals, only a domain of unknown function has been shown to be essential for the silencing function of MOM1 (Čaikovski *et al*, 2008).

So far, only a few endogenous targets of MOM1 have been identified (Steimer *et al*, 2000; Habu *et al*, 2006; Vaillant *et al*, 2006). A group of sequences called *transcriptionally silent information* (*TSI*), which represent remnants of the *gypsy*-like retrotransposon *Athila*, are transcriptionally activated in *mom1* (Steimer *et al*, 2000). Transcripts initiated in a *Mutator*-like element (*MULE-F19G14*) are also up-regulated in *mom1* (Habu *et al*, 2006). The direction of the transcripts in *MULE-F19G14* is inversed relative to the original direction of the element, and the resulting antisense transcripts contain sequences from a downstream gene (*CYCLOPHILIN40*, *CYP40*) as read-through transcripts. Notably, the region surrounding the transcription start site in *MULE-F19G14* contains both H3K4me2 and H3K9me2, normally characteristic of transcriptionally active and inactive chromatin, respectively. This indicates that the promoter region of *MULE-F19G14* has intermediate chromatin properties distinct from those of typical heterochromatin and euchromatin loci (Habu *et al*, 2006). A mutant of *DECREASE IN DNA METHYLATION1* (*DDM1*), which is responsible for the maintenance of cytosine and H3K9 methylation in heterochromatin regions (Lippman *et al*, 2004), also releases silencing of *TSI* and *MULE-F19G14*, but transcription of two other transposons analysed (*Tar17* and *AtMu1*), although active in *ddm1*, is inactive in *mom1* (Steimer *et al*, 2000; Habu *et al*, 2006). DDM1 has been proposed to recognize its targets by means of siRNAs homologous to the target sequences (Lippman *et al*, 2004), and overlapping of endogenous targets of MOM1 with those of DDM1 suggests that MOM1 might have a similar targeting mechanism, but only to a subset of loci regulated by DDM1.

In this study, towards understanding the mechanism of TGS mediated by MOM1, we performed a global comparison of RNA accumulation between the wild-type plant and *mom1* using a genome-tiling array (Matsui *et al*, 2008). The majority of up-regulated loci in *mom1* carry remnants of transposons, mostly those of *gypsy*-like retrotransposons, and are clustered around the centromeric heterochromatin regions and the heterochromatin knob on chromosome 4. In addition, our data show that *MOM1* is required for silencing of the single-copy gene, *SUPPRESSOR OF drm1 drm2 cmt3* (*SDC*), which is not related to transposons and is silenced through non-CG methylation at tandem repeats in the promoter (Henderson and Jacobsen, 2008). Furthermore, we present data indicating that MOM1 transduces RdDM signals to repressive histone modification in genomic regions showing a characteristic dependency of CG methylation on non-CG methylation.

Results

Genome-wide detection of up-regulated loci in mom1

Statistical treatment of data obtained from a genome tiling array detected 83 loci that accumulate increased levels of RNA in *mom1* (Supplementary Tables I and II). Of these 83 loci, 42 are annotated genes (hereafter referred to as AGI-genes) and the other 41 are intergenic non-AGI transcription units (non-AGI TUs; Figure 1A). No locus accumulating reduced levels of RNA in *mom1* was detected with our criteria (see Materials and methods), and no known tasiRNA or miRNA loci were found in MOM1-targets (data not shown). In the *mom1* locus, which carries at least seven copies of complex T-DNA insertions in exon 2 (YH, unpublished data, 2009), up-regulation of antisense RNA accumulation was detected in a region flanking the T-DNA insertion site (data not shown). This is likely due to read-through transcription from the integrated T-DNA and was therefore excluded from further analysis. An earlier detected MOM1-target, *MULE-F19G14/CYP40* (from *At2g15810* to *At2g15790*; Habu *et al*, 2006) was also detected in the current analysis (Supplementary Tables I and II). Selected loci were subjected to RT–PCR and their up-regulation in *mom1* confirmed (Supplementary Figures 1 and 2).

A striking characteristic of MOM1-targets is that the majority of both AGI genes and non-AGI TUs contain sequences related to transposons (Figure 1A). Furthermore, a considerable fraction of the up-regulated AGI genes (19/42 loci) and non-AGI TUs (31/41 loci) contain sequences related to *gypsy*-like retrotransposons at a frequency significantly higher than that observed in 1000 sets of reference loci consisting of randomly selected mixtures of AGI-genes and intergenic regions (Figure 1B; Materials and methods). Sequences related to the DNA transposon *Harbinger*, which is distantly related to insertion elements in eubacteria (Kapitonov and Jurka, 1999), were also detected with a significantly higher frequency (Figure 1B). On the other hand, sequences related to *Helitron*, a family of DNA transposons that transposes as rolling circle replicons (Kapitonov and Jurka, 2001), were detected with a significantly lower frequency in MOM1-targets. No significant difference in the frequency of sequences related to other transposons was observed between MOM1-targets and the random sets.

The presence of transposon sequences in the upstream and downstream regions of each MOM1-target was also surveyed. Sequences related to *En/spm* were found in the downstream region of MOM1-targets with a significantly higher frequency, and *Harbinger* was found at a higher frequency only in the downstream regions (Figure 1C). Interestingly, sequences related to *MuDR* were found in both the upstream and downstream regions of MOM1-targets at higher frequencies but not in the transcribed region. A similar but less frequent distribution was observed for *Helitron*, which exhibited significant exclusion from the transcribed region of MOM1-targets as described above (Figure 1C). The preferential targeting of MOM1 to a subset of transposons with significant biases in positions relative to the transposon sequences indicates that the targets of silencing by MOM1 could have

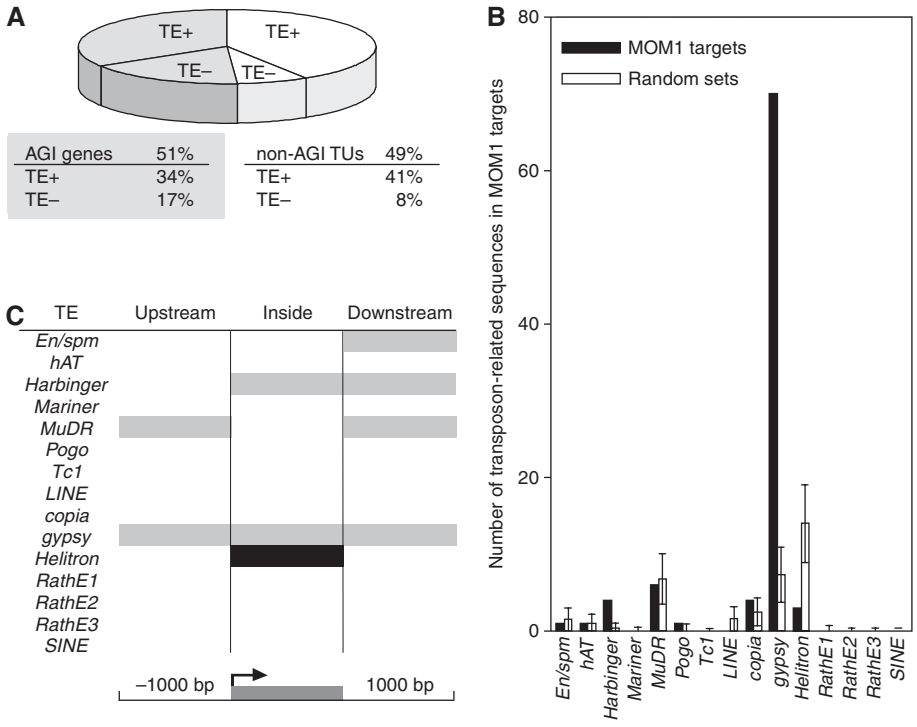

Figure 1 Characteristics of up-regulated loci detected in tiling array analysis of *mom1*. (**A**) AGI genes and non-AGI TUs in loci up-regulated in *mom1*. Forty-two AGI genes (grey, 51%) and 41 non-AGI TUs (white, 49%) are up-regulated in *mom1*. Percentages of genes and TUs-containing sequences related to transposons (TE) are indicated as TE+, whereas those without TE sequences are denoted as TE−. (**B**) Presence of transposon-related sequences in MOM1-targets. The transcribed regions of MOM1-targets were surveyed for transposon sequences, and the number of each transposon family sequences in MOM1-targets is indicated (black bars). As controls, 1000 sets of 83 randomly selected genomic regions consisting of 42 AGI genes and 41 intergenic regions with a similar size distribution to that of MOM1-targets were surveyed, and the average and standard deviation are shown (white bars). (**C**) Distribution of transposon sequences in and around MOM1-targets. Transposon sequences in regions 1-kb upstream and downstream of the transcribed regions (inside) of MOM1-targets were surveyed. Regions containing transposon sequences significantly ($P<0.05$) higher or lower than that of the random set are shown in grey and black, respectively. For examination of statistical significance, normal distribution was applied for *MuDR*, *gypsy*, and *Helitron*, and Poisson distribution was applied for others.

unknown characteristics that are not sequence specific but rather related to structure or function of these transposons.

The *gypsy*-like retrotransposon *Athila* is the most predominant repetitive element in the heterochromatin knob (Fransz *et al*, 2000; CSHL/WUGSC/PEB *Arabidopsis* Sequencing Consortium, 2000) and is found exclusively in heterochromatin regions in the genome of *A. thaliana* (Pelissier *et al*, 1996). The frequent appearance of sequences related to *gypsy*-like retrotransposons in and around MOM1-targets led us to compare the distribution of MOM1-targets with those of other transposons on the five chromosomes of *A. thaliana*. As shown earlier (Wright *et al*, 2003), *gypsy*-like retrotransposons cluster at the pericentromeric heterochromatin regions (Figure 2A). In contrast, *Helitron* and *MuDR* show a bias towards centromeres, but, being excluded, form the *gypsy*-rich pericentromeric regions. *Harbinger* distributes throughout the chromosomes with a slight bias towards centromeres. The majority of MOM1-targets have a distribution similar to that of *gypsy*-like retrotransposons, indicating that the majority of MOM1-targets are located in the pericentromeric heterochromatin. We also examined the distribution of MOM1-targets around the heterochromatin knob on chromosome 4; the heterochromatin knob is rich in transposons and is surrounded by euchromatin regions (McCombie *et al*, 2000). It has been suggested that the heterochromatin knob was created by an inversion event in which part of pericentromeric heterochromatin region was translocated to a distal region on the same chromosome (Fransz *et al*, 2000). MOM1-targets are concentrated at the cytologically defined centromeric heterochromatin region and the heterochromatin knob on the short arm of chromosome 4 (Figure 2B; Fransz *et al*, 2000; Lippman *et al*, 2004), confirming the preferential targeting of MOM1 to heterochromatin regions. It should be noted that although *Harbinger* shows only a weak concentration around centromeres (Figure 2A), the frequency of its co-localization with MOM1-targets is significantly high (Figure 1B and C). In contrast, *Helitron* shows a pattern of distribution similar to that of *MuDR* (Figure 2A), but its frequency in MOM1-targets is significantly lower than that of the random sets (Figure 1B and C). These results again suggest that targeting of MOM1 to its targets is not merely position dependent on chromosomes but rather there seem to be other unknown characteristics that are targeted by MOM1.

Involvement of siRNA pathways in silencing of MOM1-targets

Clustering of MOM1-targets at the pericentromeric–heterochromatin regions directed us to examine the presence of siRNA loci within and around MOM1-targets (Figure 3). Overlap of MOM1-targets with cloned siRNA sequences was examined using databases of the Arabidopsis Small RNA Project (Gustafson *et al*, 2005; Kasschau *et al*, 2007).

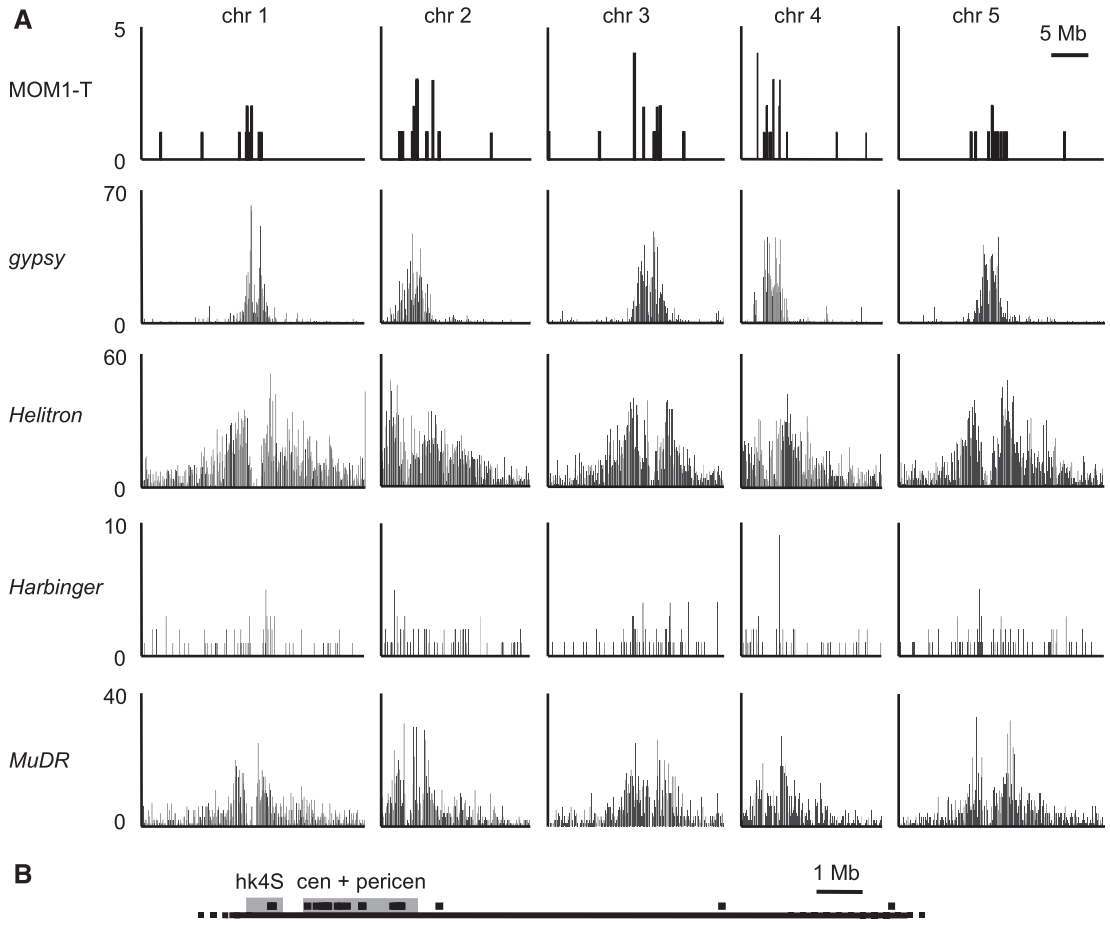

Figure 2 Distribution of MOM1-targets and transposons on each chromosome. (**A**) Chromosome distribution of MOM1-targets. The number of MOM1-targets (MOM1-T) present in each 100-kb section of each of the five *Arabidopsis thaliana* chromosomes is shown along with the corresponding number of selected transposons (*gypsy*, *Helitron*, *Harbinger*, and *MuDR*). (**B**) Distribution of MOM1-targets on chromosome 4. Positions of MOM1-targets are shown as black squares. Positions of the heterochromatin knob (hk4S) and the centromeric + pericentromeric region (cen + pericen) are shown as grey boxes.

In MOM1-targets, sequences identical to 24 nt siRNAs cloned in the wild-type plant are much more abundant than those of 21 nt siRNAs, and most of the siRNAs homologous to MOM1-targets are lost in *rdr2*, indicating that MOM1-targets contain siRNAs sequences produced in RDR2-dependent pathways. Although DCL3 works cooperatively with RDR2 to generate 24 nt siRNAs of transposons and retroelements (Vaucheret, 2006), no apparent effect of DCL3 deficiency on the abundance of the small RNAs homologous to MOM1-targets is observed. This concurs with earlier reports showing that production of siRNAs derived from transposons in *dcl3* is complemented by other DCLs (Henderson *et al*, 2006; Kasschau *et al*, 2007). Therefore, the results suggest that MOM1-targets consist of a particular set of loci possibly targeted by RdDM.

All the MOM1-targets analysed so far are also up-regulated in *ddm1* (Figure 4; Supplementary Figure 1; Steimer *et al*, 2000; Habu *et al*, 2006), further suggesting the involvement of siRNAs in silencing of MOM1-targets (Lippman *et al*, 2004). Therefore, we examined the effect of deficiency of various RNAi components on silencing of MOM1-targets (Figure 4). RT–PCR detected a low level of *TSI* RNA in the wild-type plant but not in a mutant of a gene encoding the largest subunit of RNA polymerase V (*NRPE1*; Wierzbicki *et al*, 2008), suggesting that accumulation of the low level of *TSI* transcripts in the wild-type plant depends on RNA polymerase V (Wierzbicki *et al*, 2008). In contrast, *rdr2*, but not a mutant of *RNA-DEPENDENT RNA POLYMERASE6* (*RDR6*), accumulates an elevated level of *TSI* RNA. Together with the accumulation of 24 nt siRNAs homologous to MOM1-targets in the wild type, and the prevailing appearance of sequences related to *gypsy*-like retrotransposons in MOM1-targets (Figure 1B), release of *TSI* silencing in *rdr2* strongly suggests that the majority of MOM1-targets are under the control of the siRNA pathway for heterochromatin silencing (Vaucheret, 2006). However, *dc*, a double mutant of *drm2* and *cmt3* (Johnson *et al*, 2007), did not up-regulate *TSI* (Figure 4). This indicates that being deficient in non-CG methylation is not critical for the maintenance of *TSI* silencing, but rather that other mechanisms compensate for the deficiency of non-CG methylation to maintain *TSI* silencing. Interestingly, neither *dc* nor *rdr2* releases silencing of *MULE-F19G14*, but *nrpd1* (a mutant of *NRPD1* encoding the largest subunit of RNA polymerase IV; Wierzbicki *et al*, 2008) does. Transcription of *MULE-F19G14* in *mom1* is initiated in a region that is unique in the genome, and the region around the transcription start site carries a low level of CHH methylation (Supplementary Table III; Habu *et al*, 2006) that still

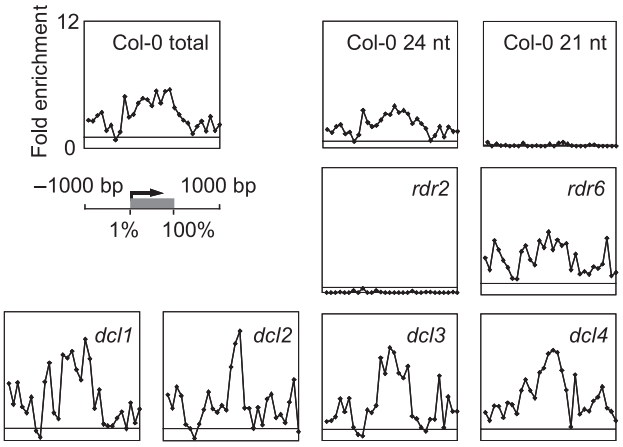

Figure 3 siRNA sequences in MOM1-targets. Sequences of siRNAs in inflorescences of the wild type and various mutants were retrieved from databases of the *Arabidopsis* Small RNA Project. Regions covering 1-kb upstream and downstream of MOM1-targets were divided into 10 sections of 100 bp, and the transcribed regions were divided evenly into 10 sections regardless of length. The counts were normalized to their copy number in the genome and the total count of small RNAs in each library. One thousand sets of randomly chosen genomic sequences were analysed as controls. In each panel, a thin horizontal line indicates the relative level of the siRNA counts in the random set (see Materials and methods for details).

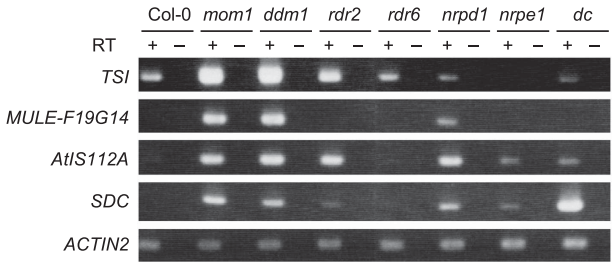

Figure 4 Differential effects of mutations in RNA silencing on MOM1-targets. Wild type (Col-0) and various mutants deficient in RNA silencing and DNA methylation (*mom1*, *ddm1*, *rdr2*, *rdr6*, *nrpd1*, *nrpe1*, and *dc*) were examined for activation of representative MOM1-targets (*TSI*, *MULE-F19G14*, *AtIS112A*, and *SDC*). *ACTIN2* was used as a control. Primers used are listed in Supplementary Table IV. RT–PCR for *TSI* was performed for 40 cycles to detect the weak signal in the wild-type plant, and therefore the intensities of the strong signals amplified from *mom1* and *ddm1* are saturated here.

accumulates siRNAs (Lister *et al*, 2008). These observations indicate that silencing of *MULE-F19G14* depends on siRNA pathways that are not typical of siRNA-mediated heterochromatin silencing (Vaucheret, 2006; Mosher *et al*, 2008). *AtIS112A* (*At4g04293*), a transposon-related pseudogene, and *SDC* (Henderson and Jacobsen, 2008) showed a dependency on RNAi components similar to that of the typical RdDM pathway (Figure 4; Vaucheret, 2006). Taken together, the results indicate that MOM1-targets are silenced by pathways involving short RNAs, and that MOM1 silences its targets by recognizing unknown characters that are common in these loci.

mom1 releases silencing of SDC, a gene regulated by non-CG methylation on short tandem repeats, without changes in cytosine methylation and siRNA accumulation

SDC encodes an F-box-like protein and is silenced in the wild-type plant by non-CG methylation on tandem repeats consisting of seven repeating units in the promoter region (Zhang *et al*, 2006; Henderson and Jacobsen, 2008). Although activation of *SDC* causes morphological aberration (Henderson and Jacobsen, 2008), release of *SDC* silencing in *mom1* does not result in any morphological abnormalities (Amedeo *et al*, 2000; Probst *et al*, 2003). RT–PCR (Figure 4) and RNA blot analyses (Supplementary Figure 3) revealed that the level of activation of *SDC* in *dc* is much higher than that in *mom1*, indicating that the weak activation of *SDC* in *mom1* is not sufficient to cause the morphological alteration. Mutations in other genes required for RdDM of heterochromatin also reactivated *SDC*, although all of the single mutants examined here confer only weak effects on *SDC* silencing (Figure 4). A weak activation of *SDC* in *rdr2*, *nrpd1*, and *nrpe1* might be due to spreading of the primary RdDM signal from the tandem repeats to the surrounding regions, which would confer an extended level of silencing in the absence of the primary silencing signals (Henderson and Jacobsen, 2008).

Cytosine methylation by RdDM is required for the production of secondary siRNA, ensuring silencing by feedback enhancing cycles (Pikaard *et al*, 2008). Therefore, if *mom1* released silencing without affecting RdDM and siRNA accumulation, MOM1 would work downstream, or independently, of the above-mentioned silencing feedback loop. Bisulfite sequencing analysis revealed that cytosine methylation in the tandem repeat region of *SDC* is not changed in *mom1*, although, as reported earlier (Henderson and Jacobsen, 2008), *dc* erases methylation of cytosines in all three sequence contexts (Figure 5A). This suggests that MOM1 is not involved in RdDM of *SDC* tandem repeats. The absence of reduction of siRNA derived from the tandem repeats in *mom1* also supports this idea (Figure 5C). It should be noted that the frequency of CHH methylation in the wild-type plant in the tandem repeat region of the *SDC* promoter is low (∼6%; Figure 5B; Supplementary Table III), although heavy cytosine methylation at all three contexts (CG, CHG, and CHH) has been commonly observed in the regions controlled by RdDM induced by transgenes producing promoter-dsRNA (Kanno *et al*, 2004; Matzke and Birchler, 2005; Daxinger *et al*, 2009). Importantly, cytosine methylation in the CHH context upstream of the transcription start site of other MOM1-targets (*MULE-F19G14* and *AtIS112A*; Supplementary Table III and Supplementary Figure 3) is also low (∼7%, Supplementary Table III; Habu *et al*, 2006). This suggests that the silencing mechanism of MOM1-targets is distinct from that induced by promoter-dsRNA. This idea is supported by the observation that MOM1 is dispensable for RdDM induced by a promoter-dsRNA system (Aufsatz *et al*, 2002).

MOM1 is required for maintenance of intermediate levels of H3K9me2 at the core region of RdDM

In the genome of *A. thaliana*, repressive histone modification is tightly linked to CG and CHG methylation (Jackson *et al*, 2002; Tariq *et al*, 2003). However, our earlier study indicated that loci silenced by MOM1 have an intermediate chromatin state in which both active (H3K4me2) and inactive (H3K9me2) histone marks coexist regardless of the presence of cytosine methylation and their silent state in the wild-type

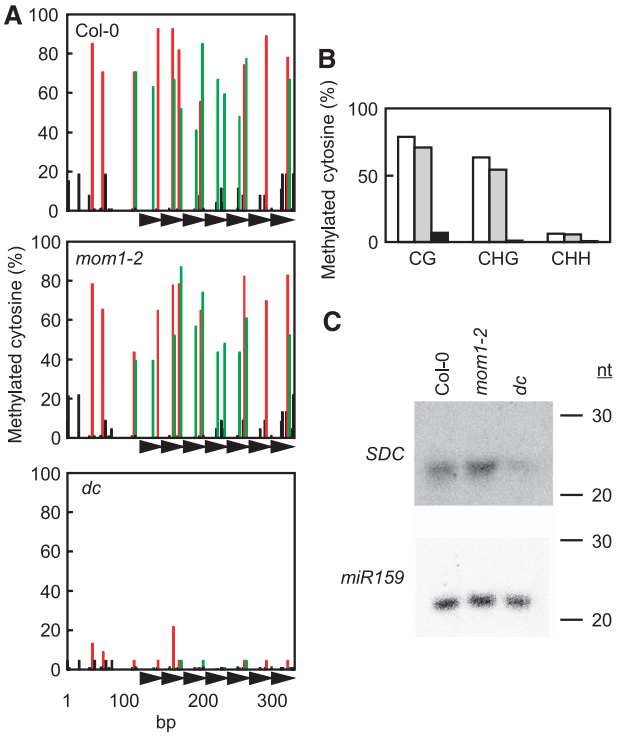

Figure 5 Cytosine methylation and siRNA accumulation in the promoter region of *SDC*. (**A**) Cytosine methylation in the *SDC* promoter. Bisulfite genomic sequencing was performed for a 334-bp promoter region of *SDC* in Col-0 (top, $n=27$), *mom1-2* (middle, $n=23$), and *dc* (bottom, $n=27$). The percentage of cytosine methylation at each position is indicated with a red (CG), green (CHG), or black (CHH) bar. The position of tandem repeats in the *SDC* promoter is indicated by black arrowheads at the bottom of each panel. (**B**) Percentage of cytosine methylation at CG, CHG, and CHH sites in the *SDC* promoter. White bars, Col-0; grey bars, *mom1*; black bars, *dc*. (**C**) Accumulation of siRNA derived from the tandem repeat region in the *SDC* promoter. A probe for *miR159* was used as a control.

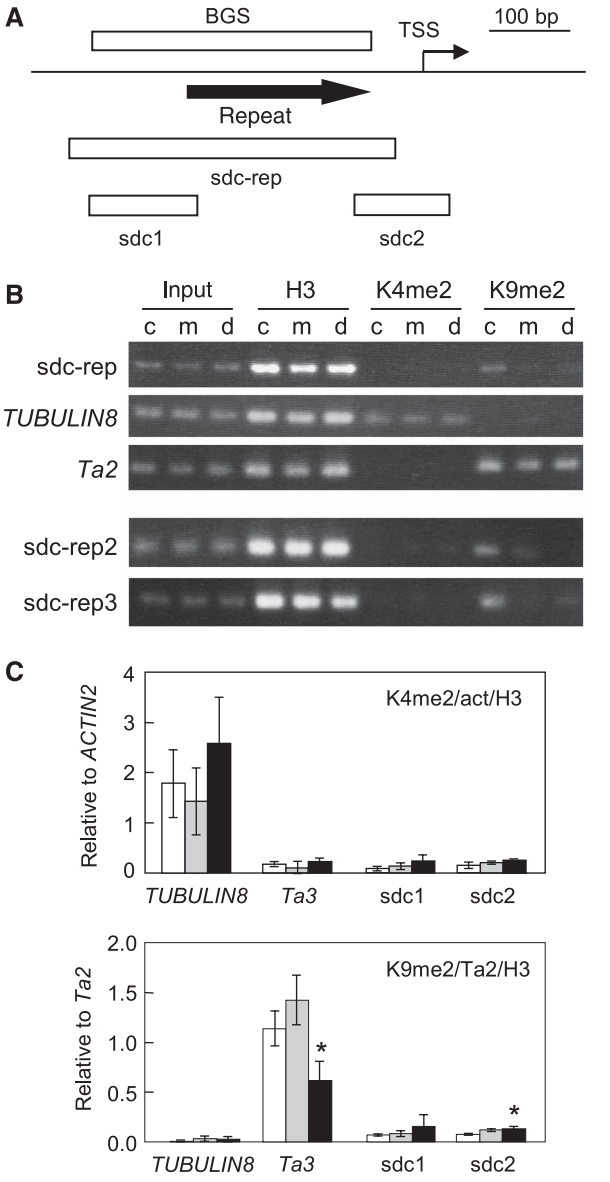

Figure 6 Histone modification around the *SDC* tandem repeats. (**A**) Schematic representation of a region around the transcription start site (TSS; Henderson and Jacobsen, 2008) of *SDC*. The tandem repeat region is indicated by a thick arrow. Regions analysed by bisulfite genomic sequencing (BGS; Figure 5) and ChIP (sdc-rep, sdc1, and sdc2) are indicated by boxes. (**B**) Histone modification at the *SDC* tandem repeats analysed with antibodies against H3K4me2 (K4me2) and H3K9me2 (K9me2). Equal amounts of input DNA (input) and those recovered from immunoprecipitates with an antibody against histone H3 C-terminus (H3) were included in the analysis. *TUBULIN8* and *Ta2* were analysed as controls for active and inactive genes, respectively. For the sdc-rep region, results of repeated experiments with independent immunoprecipitates (sdc-rep2 and sdc-rep3) are also shown. c, Col-0; m, *mom1*; d, *dc*. (**C**) Quantitative analysis of histone modification around the *SDC* tandem repeats. Data are shown as relative amounts of modification per histone H3 normalized with *ACTIN2* (for H3K4me2) or *Ta2* (for H3K9me2). Three independent immunoprecipitates for each line were subjected to quantitative PCR, and means and standard errors are shown for each target region (white, Col-0; grey, *mom1*; black, *dc*). Statistical significance in data of *mom1* or *dc* compared with Col-0 was determined by Kruskal–Wallis test and Scheffe's F test (*$P<0.05$).

plant (Habu *et al*, 2006). Chromatin immunoprecipitation (ChIP) was carried out to examine the levels of H3K4me2 and H3K9me2 in the *SDC* promoter region on activation in *mom1* (Figure 6A). In contrast to our earlier observations in *MULE-F19G14* (Habu *et al*, 2006), the *SDC* tandem repeats are relatively rich in H3K9me2 compared with H3K4me2 in the wild-type plant (Figure 6B). It should be noted that the relative intensities of the signals of H3K9me2 compared with those of H3 in Col-0 are much weaker than those of *Ta2*, indicating that the levels of H3K9me2 at the *SDC* tandem repeats are less than those of typical heterochromatin loci (Johnson *et al*, 2002). An earlier study (Henderson and Jacobsen, 2008) reported that cytosine methylation and siRNA accumulation spread from the *SDC* tandem repeats into the flanking regions. Our ChIP combined with qPCR analysis showed that the regions flanking the *SDC* tandem repeats also carry intermediate levels of H3K9me2. The results suggest that the high levels of CG and CHG methylation do not correlate directly with high levels of H3K9me2 in the *SDC* tandem repeat region that is the 'core' region of RdDM for *SDC* silencing, and that the spread of cytosine methylation and siRNA into the flanking regions is also accompanied by intermediate levels of H3K9me2. Surprisingly, the level of H3K9me2 in the tandem repeats is reduced in *mom1* (Figure 6B) regardless of the maintenance of cytosine methylation and siRNA accumulation (Figure 5A and C). A similar reduction of H3K9me2 was also observed in

dc, which loses most of its cytosine methylation in the *SDC* tandem repeats. However, in regions flanking the tandem repeats, levels of H3K9me2 in *mom1* and *dc* are not reduced. *Ta3* contains high levels of H3K9me2, which is reduced in *dc* (Figure 6C), consistent with an earlier study reporting that H3K9me2 in *Ta3* is reduced in *cmt3* (Johnson *et al*, 2002). Our data suggest that both MOM1 and non-CG methylation are required for the maintenance of H3K9me2 in the *SDC* tandem repeats, but not for the flanking regions, and that the intermediate levels of H3K9me2 in the *SDC* tandem repeats correlate with the activity of *SDC*.

To examine the involvement of MOM1 in the maintenance of the intermediate H3K9me2, we then focused on a region located 6-kb upstream of the transcription start site of *MULE-F19G14* that shows dependency of CG methylation on non-CG methylation similar to that of the *SDC* promoter (Henderson and Jacobsen, 2008; Lister *et alet al*, 2008). This region corresponds to *VANDAL18NA*, a member of the *MuDR* transposon family (Kapitonov and Jurka, 1999). A predicted gene (*At2g15815*) located between *VANDAL18NA* and *MULE-F19G14* is also activated in *mom1* (Supplementary Table I), suggesting the possibility that *VANDAL18NA* could be a core region of RdDM, and be regulated in a manner similar to that of the *SDC* tandem repeats. As multiple copies of *VANDAL* elements are present in the genome of *A. thaliana*, we designed primers for quantitative ChIP to amplify regions overlapping with two low-copy regions in *VANDAL18NA* (Figure 7A). The results of ChIP analysis showed that both regions analysed here carry intermediate levels of H3K9me2 in the wild-type plant and, in at least one of the regions, the level of H3k9me2 is significantly reduced in *mom1* and *dc* (Figure 7C). It should be noted that *VANDAL18NA* shows no increased accumulation of RNA in *mom1* (Supplementary Tables I and II) and *dc* (Lister *et al*, 2008), suggesting that the reduction of H3K9me2 is not a consequence of transcription activation. The results confirm the maintenance of intermediate levels of H3K9me2 by MOM1 in regions where CG methylation depends on non-CG methylation.

To examine further the requirement of MOM1 in the maintenance of H3K9me2 at the sites of RdDM, we analysed *AtIS112A* (*At4g04293*), which belongs to the *Harbinger* DNA family of transposons (Kapitonov and Jurka, 2003) and is strongly activated in *mom1*, in addition to mutants of components of the RdDM pathway (Figure 4). The proximal promoter region (up to −500 bp) of *AtIS112A* contains only seven CHG sites that are less methylated compared with those in the *SDC* tandem repeats in the wild-type plant, whereas the extent of CG methylation is comparable to that of *SDC* (Figure 7B and D; Supplementary Table III). Importantly, CG methylation in this region shows no dependency on non-CG methylation (Lister *et al*, 2008), suggesting that the proximal promoter region of *AtIS112A* has characteristics distinct from that of the *SDC* tandem repeats but rather similar to that of *MULE-F19G14*, which also showed no dependency of CG methylation on non-CG methylation (Habu *et al*, 2006; Lister *et al*, 2008). Bisulfite sequencing analysis showed that the pattern of cytosine methylation in the proximal promoter region of *AtIS112A* is not greatly changed on activation in *mom1* (Figure 7D), and ChIP analysis showed that this region has intermediate levels of H3K9me2 that are also unchanged in *mom1* and *dc* (Figure 7C). The results suggest that MOM1 and non-CG methylation are dispensable for the maintenance of the intermediate levels of H3K9me2 in the proximal promoter region of *AtIS112A*, and that the levels of H3K9me2 in this region do not correlate with the activity of *ATIS112A*. Together, the results indicate that MOM1 is required for the maintenance of intermediate levels of H3K9me2 at regions where non-CG methylation has an important function in the maintenance of cytosine methylation, and suggest the possibility that the site of MOM1 action is not necessarily the proximal promoter region, but can be several kilobases distant from the transcription start site.

Discussion

The majority of loci up-regulated in *mom1* contain sequences related to *gypsy*-like retrotransposons (Figure 1B) and are clustered at pericentromeric heterochromatin regions (Figure 2) and in the heterochromatin knob (Figure 2B), suggesting that MOM1 acts on regions of heterochromatin in the genome. However, MOM1-targets are not restricted to heterochromatin regions, and a minor fraction of genes located in euchromatic regions was also up-regulated in *mom1* (Figure 2). Therefore, silencing signals recognized by MOM1 are not limited to those specific to heterochromatin but are common to all the up-regulated loci including those in the euchromatic regions. Statistical analyses of the tiling array data in terms of co-localization of MOM1-targets and transposons also suggested unknown characteristics of MOM1-targets that are not merely position dependent on the chromosomes and are not restricted to specific sequences. It should be noted that MOM1-targets include *Helitron* at a significantly low frequency (Figure 1B and C). As *Helitron* is thought to target active genes undergoing transcription (Lal *et al*, 2003), its low frequency in MOM1-target sequences may reflect exclusion of MOM1 from active chromatin environments.

Kasschau *et al* (2007) reported a significant decrease of siRNA clusters within 200-bp regions flanking both the 5′ and 3′ boundaries of transcribed genes, whereas pseudogenes show no such bias. Furthermore, an additional difference between genes and pseudogenes was found in the frequency of siRNA loci in transcribed and flanking regions: in genes, the flanking regions carry higher counts of siRNA loci that are strongly reduced in *rdr2*, whereas pseudogenes exhibit similar frequencies of siRNA counts in both transcribed and flanking regions (Kasschau *et al*, 2007). The distribution pattern of siRNA loci around MOM1-targets belongs to that of neither the gene set nor the pseudogene set, but rather shows an intermediate pattern (Figure 3). These characteristics of MOM1-targets again indicate that MOM1-targets consist of a subset of loci carrying characters distinct from those of typical genes and pseudogenes.

Preferential up-regulation of genes in the pericentromeric heterochromatin was reported in a mutant of *DNA METHYLTRANSFERASE1* (*MET1*), and the distribution of the up-regulated loci in *met1* correlates well with that of methyl cytosines and repeats, including transposons (Zhang *et al*, 2006). The distribution of RNA polymerase IV-dependent siRNA loci also exhibits a similar pattern of concentration in centromeric regions (Zhang *et al*, 2007; Mosher *et al*, 2008). Although the RdDM pathway including PolIV/RDR2/DCL3/AGO4/DRM2 is thought to repress genes in hetero-

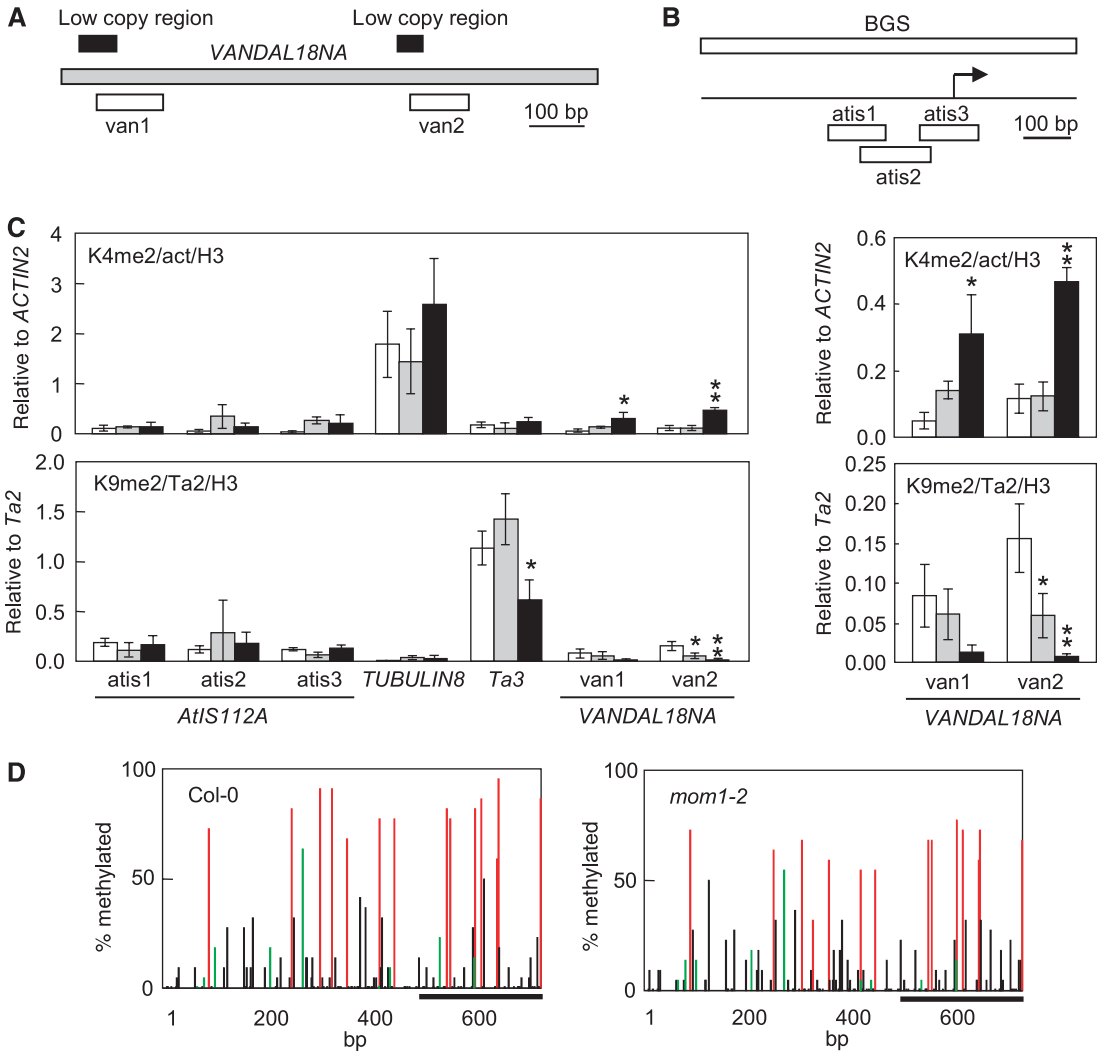

Figure 7 Cytosine and histone methylation in *AtIS112A* and *VANDAL18NA*. (**A**) Schematic representation of regions analysed by ChIP (van1 and van2) in *VANDAL18NA* (grey box). Low-copy regions are indicated by black boxes. (**B**) Schematic representation of a region around the transcription start site (bent arrow) of *AtIS112A*. Regions analysed by bisulfite genomic sequencing (BGS) and ChIP (atis1, atis2, and atis3) are indicated by boxes. (**C**) Quantitative analysis of histone modification around the transcription start site of *AtIS112A* and two regions in *VANDAL18NA*. Data are shown as relative amounts of modification per histone H3 normalized with *ACTIN2* (for H3K4me2; top left) or *Ta2* (for H3K9me2; bottom left). Three independent immunoprecipitates for each line were subjected to quantitative PCR, and means and standard errors are shown for each target region (white, Col-0; grey, *mom1*; black, *dc*). Magnified graphs for *VANDAL18NA* are shown on the right (top, H3K4me2; bottom H3K9me2). Statistical significance in data of *mom1* or *dc* compared with Col-0 was determined by Kruskal–Wallis test and Scheffe's F test (*$P<0.05$; **$P<0.01$). Data for *TUBULIN8* and *Ta3* were taken from Figure 6. (**D**) Cytosine methylation in the region shown in panel (**B**). Bisulfite genomic sequencing was performed for a 731-bp region around the transcription start site of *AtIS112A* including the 230-bp transcribed region (indicated by bars below each panel). Twenty-two independent clones were sequenced for both Col-0 and *mom1*. The percentage of cytosine methylation at each position is indicated by a red (CG), green (CHG), or black (CHH) bar.

chromatin regions (Vaucheret, 2006), *rdr2* and a triple mutant of *DRM1*, *DRM2*, and *CMT3* (*ddc*) show no bias in the distribution of up-regulated loci to pericentromeric heterochromatin (Zhang *et al*, 2006; Kurihara *et al*, 2008). Rather, up-regulated loci in *rdr2* and *ddc* are distributed throughout euchromatic regions in each chromosome and show striking differences from that of methylated cytosine and transposons. A possible explanation for this discrepancy is that the RdDM pathway functions redundantly with other pathways such as maintenance methylation of cytosine by MET1 and/or DDM1, and the extent of the contribution of these RdDM-dependent and -independent silencing pathways is locus specific (Henderson and Jacobsen, 2008; Mosher *et al*, 2008). An earlier report showed that MOM1 and DDM1 silence a subset of genomic loci in independent but mutually reinforcing pathways (Mittelsten Scheid *et al*, 2002). All of the MOM1-targets so far examined are de-repressed in *ddm1*, but several transposons activated in *ddm1* remain silent in *mom1* (Figure 4; Supplementary Figure 1; Steimer *et al*, 2000; Habu *et al*, 2006). Silencing of transposons is also mediated by DDM1, and the presence of siRNAs homologous to transposon sequences has been proposed as a possible criterion distinguishing transposons from genes (Lippman *et al*, 2003; Vaughn *et al*, 2007). Our data also show that silencing of MOM1-targets depends on the RdDM pathways but with varied requirements for their critical components (Figure 4). In addition to the transcript profiling data (Figures 1–3), the fact that there is no apparent enhancement of morphological abnormalities or RNA accumulation from selected MOM1-targets in a *mom1 drm2 cmt3* triple mutant supports the

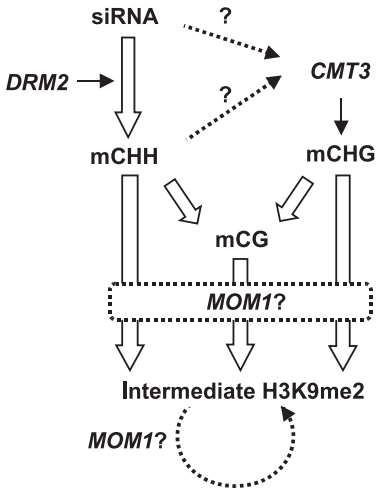

Figure 8 A model for the maintenance of intermediate levels of H3K9me2 by MOM1 at the core RdDM region. DRM2 directs CHH methylation (mCHH) guided by siRNAs in the core RdDM region, and CMT3 directs CHG methylation (mCHG) independent of or with unknown connection to RdDM. Both CHH and CHG methylation are required for CG methylation (mCG) and the intermediate H3K9me2 in the *SDC* promoter and *VANDAL18NA*. In *mom1*, all CHH, CHG, and CG methyaltion is maintained but the intermediate H3K9me2 is lost, indicating that cytosine methylation is upstream of the intermediate H3K9me2. MOM1 may transduce unknown patterns of cytosine methylation to the intermediate H3K9me2 or, alternatively, the intermediate H3K9me2 could be established by unknown histone methyltransferase(s) and MOM1 may maintain it.

function of MOM1 in the RdDM pathway (Supplementary Figure 3). Furthermore, the lack of changes in cytosine methylation and siRNA accumulation on activation in *mom1* (Figures 5 and 7; Supplementary Figures 4 and 5) indicate strongly that the site of MOM1 action in the RdDM pathway is downstream of cytosine methylation (Figure 8, see below).

Cytosine methylation may inhibit active transcription by either directly inhibiting interaction of transcription activators with their binding sites or cooperating with histone modifications to form repressive states of chromatin. In addition to our earlier results on a silent transgene locus (Amedeo *et al*, 2000) and *MULE-F19G14* (Habu *et al*, 2006), all the activated loci examined here maintain their methylated state at CG and non-CG sites in their promoter regions (Figure 5; Supplementary Table III; Supplementary Figure 4), clearly indicating that cytosine methylation itself is not the inhibitory entity precluding binding of transcription factors and/or RNA polymerases to cause silencing at these loci. Our earlier study using ChIP, amplifying a region where CG methylation does not depend on non-CG methylation (Habu *et al*, 2006; Lister *et al*, 2008), suggested that the proximal promoter region of MULE-F19G14 has an intermediate state of histone modification where no changes in H3K4me2 and H3K9me2 are seen on activation in *mom1* (Habu *et al*, 2006). However, in this study, we found that *mom1* reduces H3K9me2 in the core region of RdDM (Figures 6 and 7). *VANDAL18NA*, located 6-kb upstream of MULE-F19G14, may regulate silencing of MULE-F19G14 and one more locus between *VANDAL18NA* and MULE-F19G14 by functioning in a manner resembling an enhancer for transcription activation.

Although our data indicate a tight relationship between MOM1-targets and siRNA-mediated heterochromatin silencing (Figures 1–4), the mechanism whereby MOM1 transduces the RdDM signal to H3K9me2 is currently unknown, and we cannot exclude the possibility that MOM1 maintains H3K9me2 in the core region independently of RdDM. A reduction of H3K9me2 with a concomitant increase of H3K4me2 in *VANDAL18NA* is obvious in *dc*, whereas changes in these histone modifications in *mom1* are weak. This suggests the hypothesis that MOM1 works downstream of RdDM, independently of the KYP/CMT3 feedback loop for the maintenance of silent chromatin states (Johnson *et al*, 2007). It should be noted that putative MOM1 proteins in other plant species have chromodomains and/or plant homeodomain motifs, which are known to associate with methylated histones (Čaikovski *et al*, 2008). MOM1 is evolutionally related to the Mi-2/chromodomain-helicase-DNA binding protein 3-like chromatin remodelling proteins that are components of negative regulators of gene expression by cooperating with histone deacetylases and methyl-CpG-binding domain proteins (Bowen *et alet al*, 2004; Čaikovski *et al*, 2008). The MOM1 family of proteins in plants may participate in maintaining the silent state of loci by recognizing specific patterns of cytosine or histone modification controlled by RdDM, and thus maintain H3K9me2 (Figure 8). This idea is consistent with our earlier observations in which inducible knockdown of *MOM1* in mature leaves released TGS without DNA replication (Tariq *et al*, 2002).

Bivalent chromatin states similar to the intermediate states observed in MOM1-targets have been found in mammalian embryonic stem cells before differentiation (Bernstein *et al*, 2006), suggesting that loci carrying the intermediate state of chromatin in plants have the potential to respond to developmental and/or environmental signals. The characteristics of MOM1-targets that are distinct from typical heterochromatin and euchromatic genes, and the absence of apparent orthologues of *MOM1* in organisms other than plants, suggest the involvement of MOM1 in plant-specific events such as plasticity in cell identity and genome stability. Examination of the stability of the silent state of MOM1-targets during development and under environmental stress conditions will give hints to address the question of whether the intermediate chromatin state has any biological significance similar to that observed in mammalian embryonic stem cells.

Materials and methods

Plant materials, RNA extraction, and genome tiling array

Wild-type (Col-0) and *mom1-2* (SAIL_610_G01; Habu *et al*, 2006) plants were grown on soil and total RNA was extracted from rosette leaves of 3-week-old seedlings using Trizol (Invitrogen). Three independent RNA samples were subjected to genome tiling array analysis and the data were evaluated with *U*-test (false discovery rate <0.05) and *P* initial value ($P<10^{-8}$) as described (Matsui *et al*, 2008). Detection of intergenic transcribed units was performed as described earlier (Toyoda and Shinozaki, 2005) based on the TAIR8 annotation (http://www.arabidopsis.org/). A threefold increase or decrease in RNA accumulation was taken as an additional criterion for defining MOM1-targets. The tiling array data were deposited with GEO under the accession No. GSE13092.

ddm1-2 (Vongs *et al*, 1993), *rdr2-1* (SAIL_1277_H08; Xie *et al*, 2004), *rdr6-11* (Peragine *et al*, 2004), *nrpd1* (*nrpd1a-3*, SALK_128428; Onodera *et al*, 2005), *nrpe1* (*drd3-1*; Kanno *et al*, 2005), and *dc* (Johnson *et al*, 2007) were grown on soil and RNA was extracted as described earlier (Habu *et al*, 2006).

Transposons and siRNA sequences in MOM1-targets

Coordinates of transposons were extracted from the TAIR8 genome annotation (ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR8_genome_release/TAIR8_gff3/), and family information on transposons was downloaded from TAIR (http://www.arabidopsis.org/servlets/processor?type=transposonfamily&update_action=browse). For comparison, we created 1000 random sets of genomic sequences, each of which contained randomly selected genes (42) and intergenic regions (41); the latter did not overlap any genes in TAIR8. The length of each of the latter 41 regions was determined based on a normal distribution with a mean of 1339 bp and standard deviation of 951 bp, that is those of the non-AGI TUs in MOM1-targets. The number of transposon sequences that overlapped with each locus was counted, and the average and standard deviation of each transposon family were calculated.

To examine distributions of sequences homologous to siRNAs, sequences of small RNAs in inflorescence of the wild type and various mutants were retrieved from databases in the *Arabidopsis* Small RNA Project (http://asrp.cgrb.oregonstate.edu/db/). We divided the loci, and regions of 1-kb upstream and downstream, equally into 10 chunks, and searched for the presence of siRNA sequences in each chunk. The siRNA counts were normalized to their copy number in the genome and the total count of siRNAs in each library (Kasschau *et al*, 2007). One thousand sets of randomly chosen genomic sequences (described above) were used as controls.

For Figure 3, a copy number- and library-normalized siRNA count in a section of the MOM1-targets was divided by the normalized average count of the random sets in the corresponding section. The resulting ratio of the siRNA count ([MOM1-targets]/[random sets]) was further multiplied by a factor ([average of siRNA counts in all sections of the random sets of the analysed library]/[average of siRNA counts in all sections of the Col-0 20–25 nt library]) to reflect the relative abundance of the total siRNA counts in MOM1-targets detected in each library.

RT–PCR, bisulfite genomic sequencing, and siRNA blot

RT–PCR and bisulfite genomic sequencing were carried out as described (Habu *et al*, 2006, Saze and Kakutani, 2007, respectively). Primers used are listed in Supplementary Table IV. Sequence data of bisulfite genomic sequencing are provided in Supplementary Data Sets in FASTA format, and can be viewed using CyMATE (http://www.gmi.oeaw.ac.at/en/cymate-index/). siRNA blots were performed as described (Yoshikawa *et al*, 2005) with the LNA probe for the tandem repeat of the *SDC* promoter described in Henderson and Jacobsen (2008).

Chromatin immunoprecipitation

Chromatin and ChIP were prepared as described (Kim *et al*, 2008). Antibodies used in this study were 07-030 (Millipore, USA) against H3K4me2, CMV304;W22 (Kimura *et al*, 2008) against H3K9me2, and ab1791 (Abcam, UK) against the H3 C-terminus. The precipitates were analysed with quantitative PCR (SYBR Premix ExTaq II, Takara, Japan, and ABI Prism 7000, Applied Biosystems, USA) and the relative amount of each modification was estimated as described (Schmittgen and Livak, 2008). Primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/input.htm). Because of difficulties in designing primers for the tandem repeat region in the *SDC* promoter, a 402-bp region containing the repeats was amplified and analysed on 3% (w/v) agarose/0.5 × TBE. Primers used are listed in Supplementary Table IV.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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