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# DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*

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**We propose a model for heterochromatin assembly that links DNA methylation with histone methylation and DNA replication. The hypomethylated *Arabidopsis* mutants *ddm1* and *met1* were used to investigate the relationship between DNA methylation and chromatin organization. Both mutants show a reduction of heterochromatin due to dispersion of pericentromeric low-copy sequences away from heterochromatic chromocenters. DDM1 and MET1 control heterochromatin assembly at chromocenters by their influence on DNA maintenance (CpG) methylation and subsequent methylation of histone H3 lysine 9. In addition, DDM1 is required for deacetylation of histone H4 lysine 16. Analysis of $F_1$ hybrids between wild-type and hypomethylated mutants revealed that DNA methylation is epigenetically inherited and represents the genomic imprint that is required to maintain pericentromeric heterochromatin.**

*Keywords*: *Arabidopsis*/DDM1/heterochromatin/histone methylation/MET1

## Introduction

DNA methylation is essential for normal development of most higher eukaryotes and is involved in genomic imprinting, regulation of gene expression and defense against foreign DNA (Jost and Saluz, 1993; Finnegan *et al*., 1998). In concert with histone modifications, it contributes to chromatin remodeling (reviewed by Richards and Elgin, 2002). From fission yeast to mammals, methylation of histone H3 at lysine 9 (H3K9) is considered to be crucial for heterochromatin assembly, whereas methylation of H3 at lysine 4 (H3K4) occurs preferentially within transcriptionally competent chromatin (except for yeast rDNA, Briggs *et al*., 2001) (recently reviewed in Rice and Allis, 2001; Lachner and Jenuwein, 2002; Richards and Elgin, 2002). The interactions between DNA methylation, histone modifications and chromatin structure have mainly been studied at the molecular level for specific DNA sequences. Integrated genetic, molecular and cytological approaches can provide new insights into chromatin remodeling. For example, genome-wide H4 acetylation appeared to be tightly linked to DNA replication and possibly with post-replicative processes rather than with transcriptional activity (Jasencakova *et al*., 2000, 2001). Studies on DNA methylation and histone modifications at the nuclear level using DNA methylation mutants may elucidate the process of heterochromatin formation.

Several mutants with reduced DNA methylation levels have been isolated in *Arabidopsis*. The strongest effects on DNA methylation were found in the recessive mutants *decrease in DNA methylation1* (*ddm1*; Vongs *et al*., 1993) and *methyltransferase1* (*met1*; Finnegan *et al*., 1996; Ronemus *et al*., 1996). *DDM1* encodes a SWI/SNF-like protein, presumably a chromatin remodeling factor (Jeddeloh *et al*., 1999), while *MET1* encodes a maintenance methyltransferase (Finnegan and Kovac, 2000). They are the plant homologs of the mammalian Lsh and Dnmt1 genes, respectively (Finnegan *et al*., 1996; Dennis *et al*., 2001). In both *ddm1* and *met1*, repetitive and single-copy sequences become hypomethylated, causing a reduction in methylation level by ~70% (Vongs *et al*., 1993; Ronemus *et al*., 1996). Remethylation of hypomethylated sequences is extremely slow or absent when *ddm1* is backcrossed to the wild type (Kakutani *et al*., 1999). The mutants are further characterized by release of transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Mittelsten Scheid *et al*., 1998; Morel *et al*., 2000) and by reactivation of some transposons (Hirochika *et al*., 2000; Singer *et al*., 2001). Morphological phenotypes of *ddm1* and *met1* include altered flower morphology and leaf shape, sterility and late flowering, and appear in the first homozygous mutant generation in *met1*, but only after several generations of inbreeding in *ddm1* (Finnegan *et al*., 1996; Kakutani *et al*., 1996; Ronemus *et al*., 1996).

The *ddm1* and *met1* mutants have been analyzed at the molecular and morphological level, but not in relation to histone modifications and heterochromatin formation. Heterochromatin in *Arabidopsis* nuclei is concentrated at DAPI-bright chromocenters that contain major tandem repeats (the centromeric 180 bp repeat and rDNA genes) and dispersed pericentromeric repeats (Maluszynska and Heslop-Harrison, 1991; Heslop-Harrison *et al*., 1999; Fransz *et al*., 2002). The latter consist mainly of transposable elements and low-copy sequences (Fransz *et al*., 2000; The Arabidopsis Genome Initiative, 2000). All repeats are strongly methylated in wild-type plants but

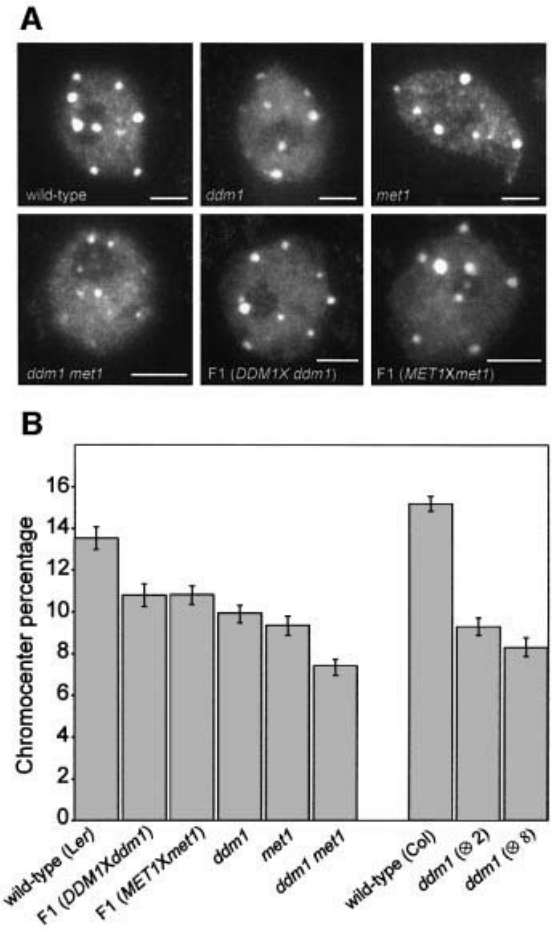



**Fig. 1.** Reduction of chromocenter size in hypomethylated mutants. (**A**) Phenotypes of representative DAPI-stained leaf interphase nuclei in a L*er* background. Chromocenters are smaller and weaker stained in *ddm1* and *met1* nuclei than in wild-type nuclei, chromocenters in the *ddm1 met1* double mutant show the weakest staining. Heterozygous *DDM1* × *ddm1* and *MET1* × *met1* $F_1$ plants show an intermediate nuclear phenotype between wild type and mutants. Bar = 5 μm. (**B**) Chromocenter fractions are shown as the percentage of area and staining intensity of chromocenters in relation to the entire nucleus. This histogram quantifies the observations shown in (A). Furthermore, it is shown that chromocenters in *ddm1* (in Col background) do not significantly reduce in size after two and eight selfing generations since the induction of the mutation. Percentages are derived from measurements of 50 nuclei each and the standard error of the mean is indicated on each bar.

weakly in *ddm1* and *met1* single mutants (Vongs *et al*., 1993; Ronemus *et al*., 1996; Kakutani *et al*., 1999).

To investigate the relationship between DNA methylation and genome-wide chromatin organization, and to elucidate the hierarchy of processes that control heterochromatin formation, we compared the location of (peri-) centromeric sequences, the nuclear patterns of DNA methylation and histone modifications, and the heterochromatin structure of leaf interphase nuclei of wild-type plants with those of *ddm1* and *met1* mutant plants.

## Results

### *Hypomethylated mutants contain reduced amounts of heterochromatin*

After DAPI staining of wild-type nuclei from the Landsberg *erecta* (L*er*) accession, conspicuous heterochromatic chromocenters can be distinguished. In nuclei of the hypomethylated mutants *ddm1* and *met1*, the chromocenters are smaller, indicating a reduction of heterochromatin (Figure 1A). This nuclear phenotype occurs in different organs, developmental stages and genetic backgrounds. We quantified this reduction of heterochromatin content by measuring the area and staining intensity of the chromocenters in relation to that of the entire nucleus (chromocenter fraction). The chromocenter fractions of *ddm1* and *met1* are reduced by ~25–30% in comparison to the wild type (Figure 1B). The double mutant *ddm1 met1* shows a further reduction of 20–25% compared with the single mutants, indicating an additive effect of both mutations for this feature. $F_1$ hybrids between wild type and either *ddm1* or *met1* mutants contain nuclei with intermediate chromocenter fractions (Figure 1B), consistent with their intermediate methylation levels (Kakutani *et al*., 1999). Since the plant phenotypes in *ddm1* appear in later generations (Kakutani *et al*., 1995), we determined (in Columbia background) whether these generations also show a further reduction in the chromocenter fraction. Although nuclei from plants of the eighth generation with a strong phenotype had smaller chromocenters than nuclei from plants of the second generation without phenotype, the difference was not significant ($P = 0.122$).

**Fig. 2.** Location of repetitive and single-copy sequences in leaf interphase nuclei. (**A**) Sequences corresponding to the 180 bp centromeric pAL repeat (red) are always located at chromocenters. Sequences corresponding to the pericentromeric BAC F28D6 (green) are located at chromocenters in wild type, but yield additional dispersed signals in the single and double mutants. Similar results were obtained with other pericentromeric BACs (F17A20, F10A2 and F21I2, DDBJ/EMBL/GenBank accession Nos AF147262, AF147259 and AF147261). (**B**) Schematic representation of BAC F28D6 (top). The different sequence elements are shown in accordance with the GenBank annotation; green boxes above (A–H) indicate the position and size of different PCR fragments used as probes in FISH experiments. Red signals on the nuclei, corresponding to the location of *Athila* elements, are always located at chromocenters. Green signals, corresponding to the location of PCR fragment C, are located at chromocenters in the wild type but yield dispersed signals in *ddm1*. (**C**) All tested repetitive elements (Tat*1* from the Ty3-*gypsy* group of LTR retrotransposons, Ta*1* from the Ty1-*copia* group of LTR retrotransposons, the MITE *Emi12*, the repetitive DNA element *AthE1.4* and the chromomeric repeat ATR63) are located at chromocenters in wild-type and mutant nuclei. (**D**) The *CAC1* sequence was most frequently detected at chromocenters in the wild-type and outside chromocenters in the *ddm1* mutant nuclei (arrow). The position of *CAC1* on BAC T10J7 is indicated by a yellow box in the scheme. FISH with this BAC yielded multiple signals (red), due to the presence of repetitive elements. Green signal is from four PCR fragments (green in the scheme), amplified from a sequence adjacent to *CAC1*, and indicates its original position. This signal is masked by the strong DAPI staining of chromocenters in the left image of the same wild-type nucleus. (**E**) The *FWA* sequence was usually located outside chromocenters, as detected by FISH with two BACs (T30C3 and F14M19), adjacent to the gene, in red and a probe of 10.5 kb, covering the gene, in green. (**F**) The *SUP* sequence was usually located outside chromocenters, as detected with a BAC (K14B15) that contains *SUP*, in red and a probe of 6.7 kb, covering the gene, in green. (A–C, E and F) Nuclei from plants with L*er* background; (D) nuclei from plants with Col background. Images in black and white show DAPI-stained nuclei; color images show FISH signals on the same nuclei. Bar = 5 μm.

### *DNA hypomethylation causes dispersion of pericentromeric sequences away from chromocenters*

The reduced size of DAPI-bright chromocenters in *ddm1* and *met1* indicates that they contain less DNA than wild-type chromocenters, and the question arises which sequences are no longer within the chromocenters in *ddm1* and *met1*. We examined this by fluorescent *in situ* hybridization (FISH) using tandem and dispersed repeats, which all localize in the wild-type chromocenters. The

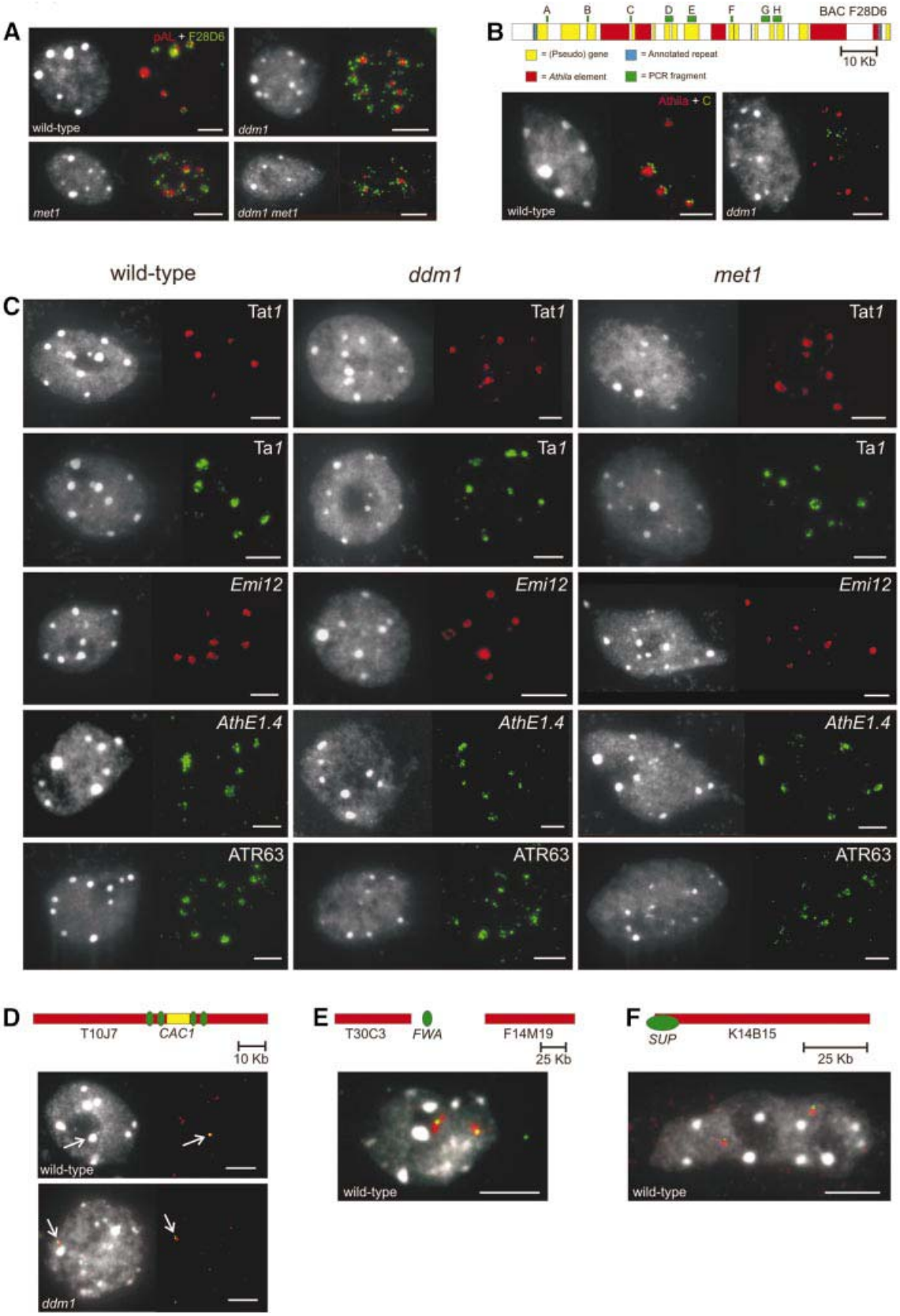


**Table I.** Number of FISH signals, present within and outside chromocenters, for different single-copy sequences and genotypes

| Gene | Accession | Genotype | No. of scored nuclei | No. of signals in chromocenters | No. of signals out of chromocenters |
|---|---|---|---|---|---|
| *CAC1* | Col | Wild type | 55 | 65 | 13 |
| *CAC1* | Col | *ddm1* | 58 | 30 | 65 |
| *FWA* | L*er* | Wild type | 104 | 24 | 154 |
| *FWA* | L*er* | *fwa-1* | 100 | 14 | 158 |
| *FWA* | Col | Wild type | 52 | 2 | 89 |
| *FWA* | Col | *ddm1* selfed 2× | 51 | 6 | 86 |
| *FWA* | Col | *ddm1* selfed 8× | 50 | 5 | 87 |
| *SUP* | L*er* | Wild type | 56 | 6 | 88 |
| *SUP* | L*er* | *clk-3* | 51 | 5 | 89 |
| *SUP* | L*er* | *ddm1* | 52 | 3 | 93 |

major tandem repeats (pAL1; see Figure 2A, 45S rDNA and 5S rDNA) co-localized with chromocenters in wild-type and mutants, and thus remained within the heterochromatin of *ddm1* and *met1* nuclei.

However, BAC DNA clones that represent sequences from the pericentromeric regions hybridized exclusively with chromocenters in the wild type, but showed a dispersed pattern at and around the chromocenters in the hypomethylated mutants (Figure 2A). This suggests that some pericentromeric sequences are located away from the chromocenter in the mutants. Most pericentromeric BAC clones contain many different transposable elements as well as non-transposable sequences (The Arabidopsis Genome Initiative, 2000). To determine which of these sequences are released from heterochromatin in the mutants, we probed wild-type and mutant nuclei with four highly repetitive elements mapped in pericentromeric regions and two (*Emi12* and *AthE1.4*) mapped in various regions along the chromosome arms. Each of these elements belongs to different families: *Athila* (Pélissier *et al*., 1995) and Tat*1* (Wright and Voytas, 1998) from the Ty3-*gypsy* group of LTR retrotransposons, Ta*1* (Voytas *et al*., 1990; Konieczny *et al*., 1991) from the Ty1-*copia* group of LTR retrotransposons, the miniature inverted-repeat transposable element (MITE) *Emi12* (Casacuberta *et al*., 1998), the repetitive element *AthE1.4* (Surzycki and Belknap, 1999) and the chromomeric repeat ATR63, which is derived from the heterochromatic knob hk4S (Fransz *et al*., 2000). All transposable elements hybridized to chromocenters in wild-type and mutant nuclei (Figure 2B and C). This implies that pericentromeric sequences other than transposable elements are relocated away from heterochromatin in the mutant nuclei. We tested this by FISH with different PCR fragments (A, B, C, D, E, F, G and H in Figure 2B) of BAC F28D6 (DDBJ/EMBL/GenBank accession No. AF147262) that represent putative genes and unannotated sequences. The fragments A, B, D, E, F, G and H contained low-copy sequences and yielded poor FISH signals outside the mutant chromocenters. However, fragment C, which has ~75 highly homologous sites in pericentromeric regions, is present at chromocenters in wild type but occupies more dispersed positions in *ddm1* and *met1* nuclei (Figure 2B). Thus, the dispersed signals from pericentromeric BACs in the hypomethylated mutants seem to be due to sequences separating the transposable elements.

The transposable elements tested above are inactive in wild type and largely inactive in the hypomethylated mutants. To find out whether the spatial position relative to heterochromatin might be related to transposon activity, we examined the location of the single-copy *CAC1* transposon located in the pericentromeric region of chromosome arm 2L, which is silent and methylated in Columbia (Col) wild-type plants but active and hypomethylated in the *ddm1* mutant (Miura *et al*., 2001). FISH with a combination of the BAC that contains *CAC1* (T10J7; DDBJ/EMBL/GenBank accession No. AC005897) and four PCR fragments, located on either side of the transposon (Figure 2D), revealed that activation and hypomethylation of the *CAC1* transposon in *ddm1* are correlated with its relocation away from the heterochromatin (Table I).

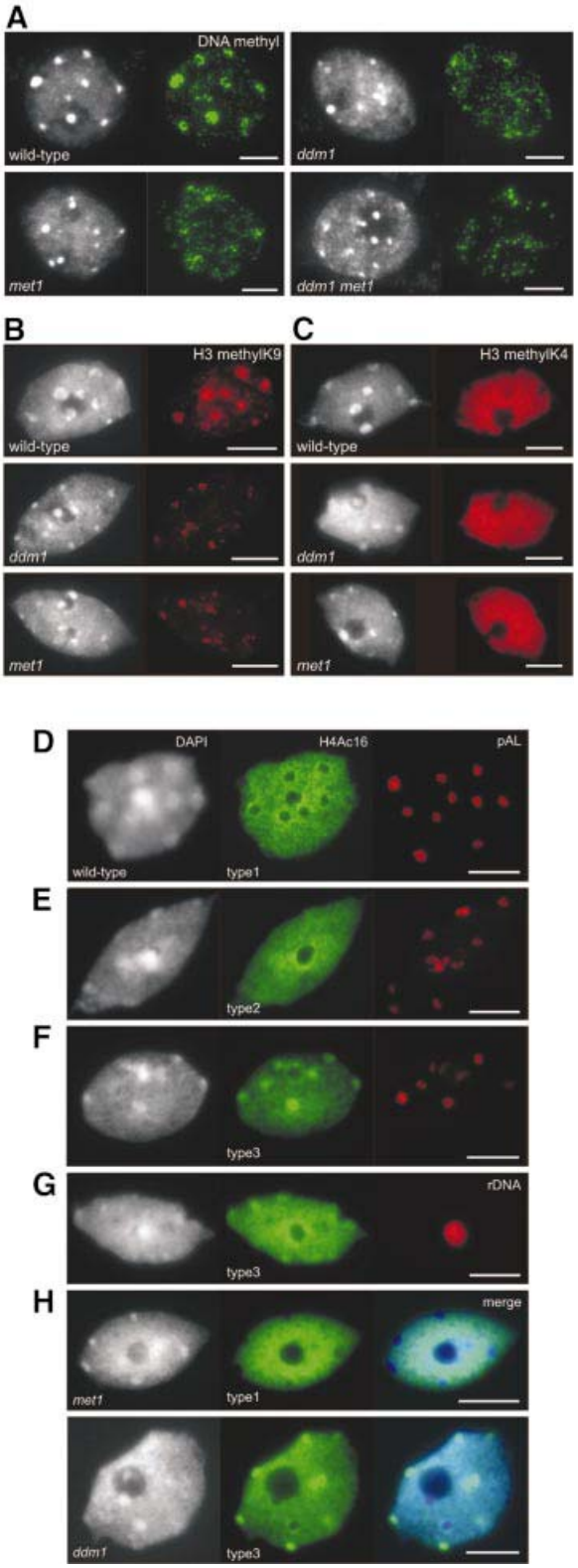
### *Not all silenced genes reside in chromocenters*

The correlation between silencing and the nuclear position of the *CAC1* transposon prompted us to investigate whether such a correlation also exists for other genes like *FWA* (mapped at the long arm of chromosome 4) and *SUPERMAN* (*SUP*; mapped at the short arm of chromosome 3), which differ in their DNA methylation and expression levels between wild type and hypomethylation mutants (Jacobsen and Meyerowitz, 1997; Soppe *et al*., 2000).

The *FWA* sequence was localized with a combination of three probes. Two BACs, positioned on either side of the gene (T30C3, DDBJ/EMBL/GenBank accession No. AL079350; and F14M19, accession No. AL049480) were detected in red and a small probe of 10.5 kb, containing *FWA*, in green (Figure 2E). In adult wild-type plants, the *FWA* gene is not expressed and the 5′-region of the gene is strongly methylated, in contrast to its hypomethylation and constitutive expression in the *fwa-1* mutant (Soppe *et al*., 2000). For both wild type and the *fwa-1* mutant, the *FWA* sequence was detected outside chromocenters in the majority of nuclei (Table I). We also compared the location of *FWA* between Col wild type (methylated and not expressed), a *ddm1* line of the second generation (methylated and not expressed) and a *ddm1* line of the eighth generation (hypomethylated and expressed). In all these genotypes, *FWA* was located mainly outside chromocenters (Table I). Therefore, silencing and

methylation of the *FWA* gene do not mediate a shift of its nuclear position toward the chromocenters.

Similar results were obtained for the *SUP* gene. In wild-type plants, *SUP* is hypomethylated and expressed in developing flowers (Sakai *et al*., 1995). Several hypermethylated alleles of *SUP* have been found [*clark kent* (*clk*) alleles], which show decreased expression (Jacobsen and Meyerowitz, 1997). The *SUP* gene is also methylated and silenced in *ddm1* and *met1* mutant plants (Jacobsen and Meyerowitz, 1997; Jacobsen *et al*., 2000). Although *SUP* is not expressed in leaves, the pattern and extent of methylation are the same in leaves and flowers (Kishimoto *et al*., 2001). The *SUP* gene was localized in leaf nuclei by FISH with two probes: the entire BAC K14B15 (DDBJ/EMBL/GenBank accession No. AB025608) containing the *SUP* gene was detected in red and a small probe of 6.7 kb, comprising the gene, in green color (Figure 2F). In all genetic backgrounds, *SUP* was preferentially located outside chromocenters (Table I).




### *Decreased DNA and H3K9 methylation accompany the size reduction of chromocenters in ddm1 and met1*

We compared the distribution patterns of methylated DNA in wild type and hypomethylated mutants using antibodies against 5-methylcytosine. Wild-type nuclei showed strong signals, especially at chromocenters (Figure 3A). In contrast, in nuclei of the *ddm1* and *met1* mutants, the immunosignals were dispersed and no longer clustered at chromocenters. This phenotype appeared to be stronger in

**Table II.** Compilation of chromatin modification data in leaf nuclei of *Arabidopsis* wild type and DNA methylation mutants

| | Wild type | *ddm1* | *met1* |
|---|---|---|---|
| H4Ac5 | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ |
| H4Ac8 | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ |
| H4Ac12 | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ |
| H4Ac16 (see Table III) | | | |
| tri-/tetra-AcH4 | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ |
| H3Ac9 | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ |
| H3 methyl K4 | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ | $eu^+$ $nu^-$ $cc^-$ |
| H3 methyl K9 | $eu^-$ $nu^-$ $cc^+$ | $eu^-$ $nu^-$ $cc^{+/-}$ | $eu^-$ $nu^-$ $cc^{+/-}$ |
| DNA methylation | $eu^-$ $nu^-$ $cc^+$ | $eu^-$ $nu^-$ $cc^{+/-}$ | $eu^-$ $nu^-$ $cc^{+/-}$ |

eu, euchromatin; nu, nucleoli; cc, chromocenter. The intensity of labeling is indicated by + or –.

**Fig. 3.** Chromatin modifications in wild-type and hypomethylated mutant nuclei. (**A**) Immunosignals for DNA methylation (green) are strongly clustered at chromocenters in wild-type nuclei; *ddm1* and *met1* nuclei have more weakly labeled chromocenters. This effect is even stronger in the double mutant *ddm1 met1*. (**B**) Histone H3 K9 methylation. In wild-type nuclei, immunosignals for H3dimethylK9 (red) localize preferentially to chromocenters, whereas *ddm1* and *met1* nuclei showed a significantly lower intensity of labeling. (**C**) Histone H3 K4 methylation (red) occurs at euchromatin, while chromocenters and nucleoli remained unlabeled in wild-type as well as in *ddm1* and *met1* nuclei. (D–G) H4Ac16 labeling patterns (green) in wild-type nuclei. Three distinct patterns can be distinguished. (**D**) Type 1: euchromatin intensely labeled, nucleoli and chromocenters unlabeled. (**E**) Type 2: chromatin more or less uniformly labeled, nucleoli unlabeled. (**F** and **G**) Type 3: chromocenters with signal clusters, nucleoli unlabeled (inactive rDNA components of chromocenters remained unlabeled in type 2 and 3 nuclei). FISH with centromeric (pAL) or 45S rDNA repeats (red, on the right). (**H**) DNA hypomethylated mutants show similar labeling patterns as wild-type nuclei. For both mutants, labeling patterns of chromocenters correlate with the reduced size of chromocenters. DAPI staining (left), immunosignals of H4Ac16 (green, middle) and the merge of both (right). All genotypes have a L*er* background. Images in black and white show DAPI-stained 3:1 in ethanol:acetic acid (A) or formaldehyde-fixed (B–H) nuclei. Adjacent color images show immunosignals on the same nuclei. Bar = 5 μm.

the double mutant *ddm1 met1*, consistent with its further reduced chromocenter fraction (Figure 3A).

Since DNA methylation has recently been reported to be tightly correlated with histone H3 methylation (for reviews, see Rice and Allis, 2001; Lachner and Jenuwein, 2002; Richards and Elgin, 2002), we analyzed the nuclear distribution of methylated histone H3 isoforms. In wild-type nuclei, immunosignals for H3methylK9 were clustered at the chromocenters. The area and intensity of H3methylK9 signals were reduced in *ddm1* and *met1* chromocenters (Figure 3B). This indicates that methylation of H3K9 is controlled by DNA methylation. Immunolabeling of H3methylK4 gave an opposite pattern that was similar for wild-type and mutant nuclei. Euchromatin was strongly labeled, while nucleoli and chromocenters (size reduced in the mutants) were unlabeled (Figure 3C). The data suggest that the decrease in DNA methylation leads to a reduction in methylated H3K9 at chromocenters.

### *H4K16 acetylation in ddm1 deviates from that of wild type and met1*

Apart from the effects of DNA methylation and histone methylation, chromatin structure is also modified by histone acetylation. Antibodies recognizing isoforms of histone H4 acetylated at lysine 5, 8 and 12, and histone H3 acetylated at lysine 9, all yielded similar patterns of immunosignals in wild-type nuclei. Euchromatin was intensely labeled, while nucleoli and heterochromatic chromocenters were unlabeled. A comparable pattern was observed in *ddm1* and *met1* nuclei (Table II), although the unlabeled domains were smaller than in wild type, correlating with the smaller chromocenters.

In contrast, antibodies against H4Ac16 yielded three classes of labeling patterns (Figure 3D–H; Table III). Type 1, showing strongly labeled euchromatin and unlabeled chromocenters and nucleoli, comprises 66.7% of the nuclei (Figure 3D). Type 2 displayed a uniformly labeled chromatin with unlabeled nucleoli and represents 24.2% of nuclei (Figure 3E). Type 3 is characterized by chromocenters that are more intensely labeled than euchromatin, while nucleoli and chromocenters containing inactive rDNA genes remain unlabeled. This class comprises 9.1% of nuclei (Figure 3F). The H4Ac16 patterns resemble the cell cycle-dependent modulation of acetylation at this lysine position observed in root meristems of faba bean (Jasencakova *et al*., 2000). However, leaf nuclei are mitotically inactive but frequently endopolyploid (Galbraith *et al*., 1991). Therefore, the high intensity of acetylation of H4K16 might reflect a link with DNA (endo-)replication or post-replicative processes, particularly at chromocenters.

We observed comparable labeling patterns for leaf nuclei of *ddm1* and *met1* mutants (Figure 3H). However, the proportion of nuclei with labeled chromocenters (type 2 and 3) was increased somewhat in *met1* (46.8%) and drastically in *ddm1* (80.2%) compared with the wild type (33.3%; Table III). This indicates that deacetylation of H4K16 depends on DDM1 activity and implies a functional difference between DDM1 and MET1 with respect to histone acetylation.

### *DNA methylation and histone H3K9 methylation, but not histone H4K16 acetylation patterns, are inherited epigenetically*

Inheritance of *ddm1*-induced DNA hypomethylation is stable, even in wild-type/*DDM1* hybrid background (Kakutani *et al*., 1999). We therefore examined chromatin structure in $F_1$ plants, heterozygous for either *ddm1* or *met1*. The nuclei of heterozygotes showed two groups of chromocenters that differed strikingly in methylation level. One group displayed the wild-type morphology, whereas chromocenters of the other were similar to those of the mutants (Figure 4A). The difference in parental origin of chromocenters was supported by FISH with 45S rDNA. This probe hybridized to four chromocenters, of which two were heavily methylated, whereas the other two were not (data not shown). This means that the DDM1 and MET1 activities in the $F_1$ nuclei do not restore the wild-type level of DNA methylation in mutant-derived chromocenters. Considering the recessive nature of the *ddm1* and *met1* mutations (Vongs *et al*., 1993; E.Richards, personal communication), this indicates that DNA methylation is inherited epigenetically. When the pericentromeric BAC F28D6 was probed to *DDM1ddm1* or *MET1met1* $F_1$ nuclei, one half of the chromocenters showed the wild-type pattern and the other half showed the mutant pattern with more dispersed signals (Figure 4B). This indicates that the formation of pericentromeric heterochromatin requires DNA methylation as an epigenetic imprint.

Combined immunolabeling experiments for DNA and H3K9 methylation on *DDM1ddm1* $F_1$ nuclei showed that strong methylation of H3K9 is restricted to chromocenters containing a high level of methylated DNA (Figure 4C). This confirms that the epigenetically inherited methylation status of DNA is responsible for the reduction in methylation of H3K9 in *ddm1*-derived chromocenters. Contrary to this, strong acetylation of H4K16 at all chromocenters occurred with similar frequencies in the *DDM1ddm1* $F_1$ as in wild type (Table III), which demonstrates that DDM1 in heterozygous plants mediates deacetylation of H4K16 but is not able to mediate remethylation of DNA once the methylation is lost.

## Discussion

### *Genome-wide DNA hypomethylation alters chromatin organization within the nucleus*

We have demonstrated that two functionally different hypomethylated mutants display the same nuclear phenotype characterized by size-reduced chromocenters with decreased levels of DNA and H3K9 methylation, and relocation of low-copy pericentromeric sequences away from the chromocenters. The euchromatic location of single-copy genes (*SUP* and *FWA*) is not affected by changes in methylation level. Although the DNA methylation level of tandem repeats and high-copy transposons is strongly reduced, the remaining DNA methylation (Lindroth *et al*., 2001) seems to be sufficient for residual heterochromatin formation. In accordance with this, the *ddm1 met1* double mutant shows less DNA methylation than the single mutants and has smaller chromocenters (Figures 1 and 3A). The remaining methylation in the double mutant is probably due to the activity of other

**Table III.** H4Ac16 labeling patterns of leaf nuclei[a] of *Arabidopsis* wild type and DNA methylation mutants

| Labeling patterns[b] | Wild type | | *ddm1* | | *met1* | | $F_1$ (L*er* × *ddm1*) | |
|---|---|---|---|---|---|---|---|---|
| | *n* | % | *n* | % | *n* | % | *n* | % |
| 1 $nu^{-}$ $cc^{-}$ $eu^{+}$ | 132 | 66.7 | 35 | 19.8 | 50 | 53.2 | 79 | 71.2 |
| 2 $nu^{-}$ $cc^{+}$ $eu^{+(+)}$ | 48 | 24.2 | 95 | 53.7 | 43 | 45.7 | 23 | 20.7 |
| 3 $nu^{-}$ $cc^{++}$ $eu^{+}$ | 18 | 9.1 | 47 | 26.5 | 1 | 1.1 | 9 | 8.1 |
| 2+3 | 66 | 33.3 | 142 | 80.2[c] | 44 | 46.8[d] | 32 | 28.8 |
| Σ | 198 | 100.0 | 177 | 100.0 | 94 | 100.0 | 111 | 100.0 |

[a]4C nuclei as the major fraction of leaf nuclei, 2C and 8C nuclei showed similar results.
[b]nu, nucleoli and rDNA component of cc.
[c]$P < 0.001$.
[d]$P = 0.037$.

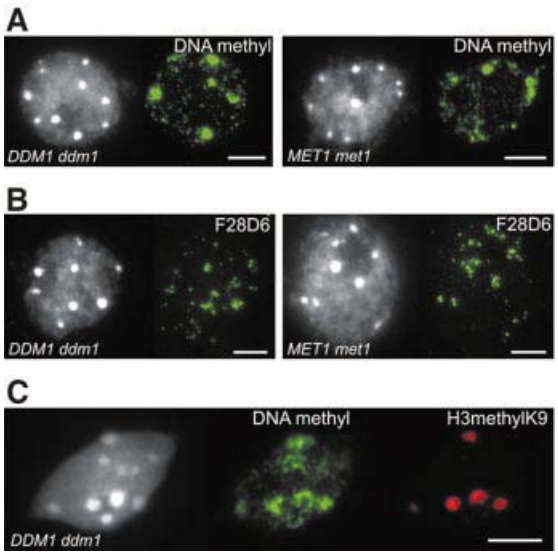



**Fig. 4.** The epigenetic inheritance of DNA methylation and H3K9 methylation is visible in nuclei of $F_1$ plants (wild type × mutant). (**A**) Half of the chromocenters show strong immunosignals for DNA methylation and the other half weak signals. (**B**) FISH signals for BAC F28D6 (green) are strongly clustered at half of the chromocenters but more dispersed around the other half. (**C**) The chromocenters with strong immunosignals for DNA methylation also show strong signals of H3K9 methylation. All genotypes have a L*er* background. Images in black and white show DAPI-stained 3:1 in ethanol:acetic acid (A and B) or formaldehyde-fixed (C) nuclei. Immunosignals are in green for DNA methylation (A and C) and in red for H3K9 methylation (C). Bar = 5 μm.

methyltransferases that partially take over the function of *MET1* (Genger *et al*., 1999). Alternatively, methylated DNA might not be required for heterochromatin formation and a critical amount of high-copy repeats might be sufficient, as in *Drosophila*, which lacks extensive DNA methylation (Henikoff, 2000). However, the relocation of low-copy pericentromeric sequences into euchromatin within nuclei of hypomethylated mutants suggests that DNA methylation is at least required for spreading of heterochromatic features into pericentromeric regions.

Unpublished data (A.Probst, O.Mittelsten Scheid, P.Fransz and J.Paszkowski, in preparation) obtained for another allele of *ddm1* indicate that even large amounts of centromeric tandem repeats are dispersed, nearly dissolving the chromocenters.

### *DNA maintenance methylation precedes H3K9 methylation*

The decrease in DNA methylation in both hypomethylated mutants is paralleled by reduced methylation of H3K9 at chromocenters. Because only maintenance DNA methylation (mainly at CpG sites) is disturbed in the *met1* mutant, the primary cause of reduction of H3K9 methylation should be the reduction in DNA methylation. In agreement with this assumption, in $F_1$ plants heterozygous for *ddm1*, only chromocenters that have reduced DNA methylation also have reduced methylation of H3K9. Therefore, we propose that maintenance CpG methylation directs histone H3K9 methylation. This seems to contradict previous results in *Neurospora crassa* (Tamaru and Selker, 2001) and *Arabidopsis* (Jackson *et al*., 2002), which show that DNA methylation is dependent on H3K9 methylation, and disagrees with the suggestion of Gendrel *et al*. (2002) that the loss of DNA methylation in *ddm1* might be a consequence of reduced H3K9 methylation in heterochromatin. This discrepancy might be explained by a difference in function between the methylases involved because the histone methylation-dependent chromomethylase (CMT3) of *Arabidopsis* specifically methylates non-CpG sites (Lindroth *et al*., 2001). If CpG DNA methylation induces H3K9 methylation and this, in turn, induces CpNpG methylation, the positive feedback might induce spreading of heterochromatin from high-copy repeats to low-copy pericentromeric sequences.

Our conclusion that maintenance DNA methylation precedes H3K9 methylation concerns the assembly of wild-type heterochromatic chromocenters. In a chromatin immunoprecipitation study of H3K9 methylation in the *ddm1* and *met1* mutants, Johnson *et al*. (2002) found a loss of H3K9 methylation in *ddm1*, but found that the majority of H3K9 methylation was retained in the *met1* mutant. In contrast, using immunolabeling experiments, we observed similar losses of H3K9 methylation signals at chromocenters in both the *ddm1* and the *met1* mutants. One possible explanation for this difference is that the particular sequences assayed by chromatin immunoprecipitation may not have shown the same global loss that we observed by immunolabeling studies. A second possibility is that the loss of H3K9 methylation immunosignals is in part due to dislocation of pericentromeric regions away from the chromocenters, as well as an actual loss of H3K9 methylation from the chromatin. However, the reduction of H3K9 methylation at remnant chromocenters (largely

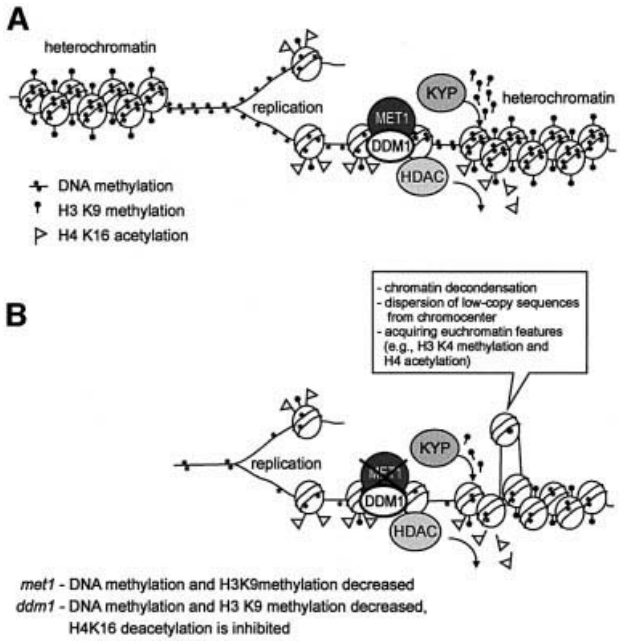



**Fig. 5.** A model for heterochromatin assembly. (**A**) In wild-type *Arabidopsis* nuclei, strong methylation of DNA and H3K9, followed by histone deacetylation, leads after replication to re-establishment of heterochromatin by DDM1, MET1, a histone H3K9-specific methylase (KYP) and a histone deacetylase. DNA maintenance methylation mediated by MET1 and supported by DDM1 directs H3K9 methylation. H4K16 deacetylation at newly replicated nucleosomes is mediated by DDM1. (**B**) In the *met1* mutant, maintenance DNA methylation is severely reduced, leading to decreased H3K9 methylation. Below a certain threshold, low-copy sequences disperse from heterochromatin and acquire euchromatin features. In the absence of DDM1, H4K16 deacetylation is additionally impaired.

composed of transcriptionally inactive sequences) in *met1* mutants seems most likely to be a direct loss of H3K9 methylation and not due to dislocation.

### *DDM1 is required for maintenance methylation activity by MET1 and additionally for deacetylation of H4K16*

Genomic DNA methylation patterns are maintained immediately after replication by the activities of DNA methyltransferase(s) with a high preference for hemimethylated DNA (Bestor, 1992). The high level of H4 acetylation at this stage (Taddei *et al*., 1999; Jasencakova *et al*., 2000, 2001) might facilitate this process. The additive effect of *ddm1* and *met1* on chromocenter size reduction in the double mutant *ddm1 met1* (Figure 1B) implies that each gene controls DNA maintenance methylation by different mechanisms. Methylation of DNA by *MET1* is probably supported by chromatin remodeling factors such as *DDM1*, as proposed by Jeddeloh *et al*. (1999). This support might be more important for highly repetitive sequences in strongly condensed heterochromatic regions than for single-copy genes in euchromatic regions, since, in *ddm1* mutants, high-copy sequences have already become hypomethylated in the first generation, whereas the single-copy sequences yielding phenotypes may become hypomethylated only in later generations.

Immunolabeling of nuclei from $F_1$ hybrids heterozygous for *ddm1* or *met1* revealed the inability of DDM1 and MET1 to re-establish DNA methylation of chromocenters once it has been lost (Figure 4A). This confirms the stable inheritance of DNA hypomethylation by *ddm1* (Kakutani *et al*., 1999), and is consistent with a role of *DDM1*and *MET1* in maintenance methylation. The inability to remethylate repetitive sequences does not prevent *de novo* methylation of multicopy transgenes (Jakowitsch *et al*., 1999; Pélissier *et al*., 1999) and endogenous genes (Melquist *et al*., 1999) in *ddm1* and *met1*. It is possible that such repetitive genes require transcription to be methylated *de novo* in the process of TGS or PTGS. Transcription is lacking for most hypomethylated centromeric and pericentromeric repetitive sequences, thus preventing their *de novo* methylation.

The increased number of nuclei with H4K16 acetylation at chromocenters of *ddm1* plants indicates that DDM1, in addition to its role in DNA methylation, is also involved in histone deacetylation, presumably after completion of maintenance DNA methylation. Similarly, a DDM1-like factor (ISWI) of *Drosophila* was found to counteract H4K16 acetylation (Corona *et al*., 2002).

### *A model for the assembly of constitutive heterochromatin in Arabidopsis*

The characteristics of constitutive heterochromatin need to be preserved through cell divisions. We propose a central role for DDM1, MET1, a H3K9-specific histone methylase (KYP) and a histone deacetylase (H4K16-specific in *Arabidopsis*) in the reassembly of heterochromatin, directly after DNA replication. (A complex of DNA methyltransferase and histone deacetylase has been proposed for mammals, see Rountree *et al*., 2000.) DNA maintenance methylation at CpG sites is performed when newly replicated nucleosomes are still accessible due to acetylated H4K16. During or after maintenance methylation of DNA, H3K9 methylation, directed by methylated DNA, might complete heterochromatin assembly, including binding of HP1-like proteins (Bannister *et al*., 2001; Gaudin *et al*., 2001; Lachner *et al*., 2001) to H3K9. Then DDM1 could mediate deacetylation of H4K16 (Figure 5A). When either DDM1 or MET1 is lacking, DNA methylation is reduced, causing reduced H3K9 methylation. At pericentromeric regions with low-copy sequences, DNA methylation and H3K9 methylation can fall below a critical threshold. As a consequence, these regions acquire euchromatin features (e.g. H3methylK4) and disperse from chromocenters (Figure 5B). If DDM1 is lacking, deacetylation of H4K16 is prevented additionally.

For other eukaryotic organisms, this model has to be modified according to their post-replicative requirement for H4 acetylation. Furthermore, it has to be considered that H3K9 methylation is not sufficient to determine constitutive heterochromatin in large plant genomes (A.Houben, D.Demidov, D.Gernand, A.Meister, C.R.Leach and I.Schubert, in preparation). Most likely, the ratio between H3methylK4 and H3methylK9 is essential. Within constitutive heterochromatin, H3K4 remains (largely) unmethylated, while euchromatin contains strongly methylated H3K4, independent of the level of H3K9 methylation. Thus, constitutive heterochromatin can be molecularly identified by the presence of a

threshold amount of (usually highly methylated) tandem repeats, and an excess of methylated H3K9 over methylated H3K4 (Noma *et al*., 2001). The model can be further tested/refined using (transgenic) mutants of *Arabidopsis*, affected in histone acetylation and methylation (Tian and Chen, 2001; Jackson *et al*., 2002).

## Materials and methods

### Plant material

The *ddm1-2* and *met1* mutants were originally obtained in the Col accession and later transferred to the L*er* background by repeated backcrossing (Jacobsen *et al*., 2000). The double mutant *ddm1 met1* was created by crossing plants with the single mutations. True double mutants were identified in $F_2$ by PCR, restriction digest and DNA sequence analysis of the mutations. Plants heterozygous for *ddm1* or *met1* were obtained from the crosses *cer2* × *ddm1* and *cer2* × *met1*, respectively. The *cer2* marker (bright green stems and siliques) was used to distinguish $F_1$ plants from self-pollinated progeny. The mutants *clk-3* (Jacobsen and Meyerowitz, 1997) and *fwa-1* (Soppe *et al*., 2000) were both in the L*er* accession.

Plants were grown either in the greenhouse with a long-day regime or in a growth chamber under continuous light. Young rosette leaves, flower buds and root tips were harvested, fixed in ethanol/acetic acid (3:1) and stored at –20°C. For immunodetection of histones, the tissue was fixed in 2% formaldehyde in Tris buffer. Nuclear suspensions were produced and processed for flow sorting as described previously (Jasencakova *et al*., 2000, 2001). Nuclei were stained with DAPI.

### FISH

Probes used: BACs were obtained from the Arabidopsis Biological Resource Center. For the amplification of pericentromeric PCR fragments A–H, BAC F28D6 (DDBJ/EMBL/GenBank accession No. AF147262) was used as template (Figure 2B). The four PCR fragments used to locate *CAC1* were based on nucleotides 41 909–44 519, 46 209–48 649, 58 108–60 468 and 62 069–64 377 from BAC T10J7 (accession No. AC005897). The different PCR fragments used for FISH detection of different repetitive elements were based on nucleotides 293–2704 from the BAC T1J24 sequence (accession No. AF147263) for *Athila*, nucleotides 17–2044 from the Tat*1* sequence (accession No. AF056631), nucleotides 1269–3124 from the Ta*1-3* sequence (accession No. X13291), nucleotides 26 751–27 824 from the FCA contig fragment No. 2 (accession No. Z97337) for *Emi12*, nucleotides 30 737–32 974 from P1 clone MHK7 (accession No. AB011477) for *AthE1.4* and nucleotides 22 121–23 847 from the BAC T5H22 sequence (accession No. AF096372) for the chromomeric repeat ATR63. PCR conditions and primer sequences can be obtained from the authors on request. Probes for detection of the *SUP* and *FWA* sequences were a 6.7 kb *SUP* genomic DNA fragment cloned into pCGN1547 and a 10.5 kb genomic DNA fragment cloned into pCAMBIA 2300, respectively. The 180 bp centromeric repeat sequence was detected with pAL1 (Martínez-Zapater *et al*., 1986).

For BLAST analyses of PCR fragment sequences, NCBI BLAST2.0 was used.

FISH experiments were performed according to Schubert *et al*. (2001) with the antibodies goat anti-avidin conjugated with biotin (1:200; Vector Laboratories) and avidin conjugated with Texas Red (1:1000; Vector Laboratories) for the detection of biotin-labeled probes, and mouse anti-DIG (1:250; Roche) and goat anti-mouse conjugated with Alexa$^{488}$ (1:200; Molecular Probes) for the detection of DIG-labeled probes.

### 5-methylcytosine immunodetection

Slide preparations were baked at 60°C for 30 min, denaturated in 70% formamide, 2× SSC, 50 mM sodium phosphate pH 7.0 at 80°C for 3 min, washed in ice-cold PBS (10 mM sodium phosphate pH 7.0, 143 mM NaCl) for 5 min, incubated in 1% bovine serum albumin in PBS for 30 min at 37°C and subsequently incubated with mouse antiserum raised against 5-methylcytosine (Podesta *et al*., 1993; 1:250) in TNB (100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% blocking reagent; Roche). Mouse antibodies were detected using rabbit anti-mouse–FITC (1:1000; Sigma), followed by goat anti-rabbit–Alexa$^{488}$ (1:200; Molecular Probes).

### Histone immunodetection

Antibodies used were rabbit polyclonal antisera recognizing specifically modified lysine residues of histones H3 and H4 [R41 (H4Ac5; 1:100), R232 (H4Ac8; 1:100), R101 (H4Ac12; 1:100), R252 (H4Ac16; 1:1000), R243 (preferentially recognizing tri- and tetra-acetylated H4; 1:200) (Turner and Fellows, 1989; Turner *et al*., 1989; Belyaev *et al*., 1996; Stein *et al*., 1997; White *et al*., 1999)], anti-acetyl histone H3 (Lys9; 1:200), anti-dimethyl-histone H3 (Lys4; 1:200–1:500) and anti-dimethyl-histone H3 (Lys9; 1:100) (from Upstate).

The immunolabeling procedure for histones was as described previously (Jasencakova *et al*., 2000, 2001). After post-fixation in 4% paraformaldehyde/PBS, subsequent washes in PBS and blocking at 37°C, slides were exposed to primary antisera for 1 h at 37°C or overnight at 4°C. After washes in PBS, the incubation with secondary antibodies, goat anti-rabbit–FITC (1:80; Sigma) or goat anti-rabbit–rhodamine (1:100; Jackson ImmunoResearch Labs) was performed at 37°C. Nuclei were counterstained with DAPI (2 μg/ml, in Vectashield; Vector).

For a combined detection of H3K9 methylation and DNA methylation, nuclei were post-fixed after histone detection, denatured and incubated with mouse anti-5mC, followed by goat anti-mouse conjugated with biotin (1:600; Jackson ImmunoResearch Labs) and streptavidin conjugated with FITC (1:1000).

### Microscopy and image processing

Preparations were analyzed with a Zeiss Axiophot 2 epifluorescence microscope equipped with a cooled CCD camera (Photometrics). Fluorescence images for each fluorochrome were captured separately using the appropriate excitation filters. The images were pseudocolored, merged and processed with Adobe Photoshop software (Adobe Systems).

### Measuring of chromocenter fractions

Digital images in gray scale were analyzed with the freeware program NIH-image 1.62. Special macros were written to measure the size and average staining intensity of nuclei and chromocenters. The chromocenter value was divided by the whole nucleus value and yielded the chromocenter fraction.

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