Meiotically and Mitotically Stable Inheritance of DNA Hypomethylation Induced by *ddm1* Mutation of *Arabidopsis thaliana*

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ABSTRACT

In contrast to mammalian epigenetic phenomena, where resetting of gene expression generally occurs in each generation, epigenetic states of plant genes are often stably transmitted through generations. The Arabidopsis mutation *ddm1* causes a 70% reduction in genomic 5-methylcytosine level. We have previously shown that the *ddm1* mutation results in an accumulation of a variety of developmental abnormalities by slowly inducing heritable changes in other loci. Each of the examined *ddm1*-induced developmental abnormalities is stably transmitted even when segregated from the potentiating *ddm1* mutation. Here, the inheritance of DNA hypomethylation induced by *ddm1* was examined in outcross progeny by HPLC and Southern analyses. The results indicate that (i) *DDM1* gene function is not necessary during the gametophyte stage, (ii) *ddm1* mutation is completely recessive, and (iii) remethylation of sequences hypomethylated by the *ddm1* mutation is extremely slow or nonexistent even in wild-type *DDM1* backgrounds. The stable transmission of DNA methylation status may be related to the meiotic heritability of the *ddm1*-induced developmental abnormalities.

IN both plants and mammals, epigenetic control of gene expression is often correlated with change in cytosine methylation of the affected locus. Mammalian epigenetic phenomena, such as parental imprinting and X-chromosome inactivation, are developmentally regulated, and "resetting" of the epigenetic status occurs in each generation. Similarly, methylation patterns in mammalian genome undergo reorganization (Monk *et al.* 1987) by extensive demethylation and "*de novo*" methylation during gametogenesis and early development (Yoder *et al.* 1997). In contrast, the epigenetic states of plant genes such as the Arabidopsis *SUPERMAN* gene (Jacobsen and Meyerowitz 1997), *PAI* genes (Bender and Fink 1995), maize transposable elements (McClintock 1967; Brutnell and Dellaporta 1994; Martienssen and Baron 1994; Schlappi *et al.* 1994), and repeated transgenes of tobacco (Park *et al.* 1996) are often stably inherited through generations.

Eukaryotic mutants affecting genomic DNA methylation have been described in mouse (Li *et al.* 1992), Neurospora (Foss *et al.* 1993), Ascobolus (Malagnac *et al.* 1997), and Arabidopsis (Vongs *et al.* 1993; Finnegan *et al.* 1996; Ronemus *et al.* 1996; Mittelsten-Scheid *et al.* 1998). As in other eukaryotes (Li *et al.* 1992; Foss *et al.* 1993; Malagnac *et al.* 1997), developmental abnormalities were exhibited in the Arabidopsis DNA methylation mutants. In homozygous *ddm1* mutants of Arabidopsis, genomic 5-methylcytosine (5mC) content in *Taq*I sites is reduced to ~30% of wild-type levels (Vongs *et al.* 1993). The *ddm1* mutations result in an accumulation of a variety of developmental abnormalities, by inducing heritable changes in other loci. Each of the *ddm1*-induced developmental abnormalities investigated was stably transmitted even when segregated from the potentiating *ddm1* mutation (Kakutani *et al.* 1996; Kakutani 1997). A similar spectrum of developmental abnormalities was found in transgenic plants expressing a DNA methyltransferase gene *MET1* (Finnegan and Dennis 1993) in an antisense orientation (Finnegan *et al.* 1996; Ronemus *et al.* 1996).

In addition to revealing effects of altering DNA modification on development, DNA methylation mutants provide good systems with which to investigate *de novo* methylation *in vivo*. For example, disruption of a mouse DNA methyltransferase gene (*Dnmt1*) causes a reduction in overall DNA methylation levels (Li *et al.* 1992). Expression of the wild-type *Dnmt1* cDNA in mutant male embryonic stem (ES) cells causes an increase in methylation of bulk DNA to normal levels, while restoration of the methylation of the imprinted genes *H19* and *Igf2r* occurs only after germline transmission (Tucker *et al.* 1996). These results suggest the existence of *de novo* methyltransferase activities specific during oogenesis and spermatogenesis.

We have previously proposed that remethylation of sequences hypomethylated by *ddm1* mutations is slow, on the basis of the following observations using thin-layer chromatography (Vongs *et al.* 1993): (i) Heterozy-

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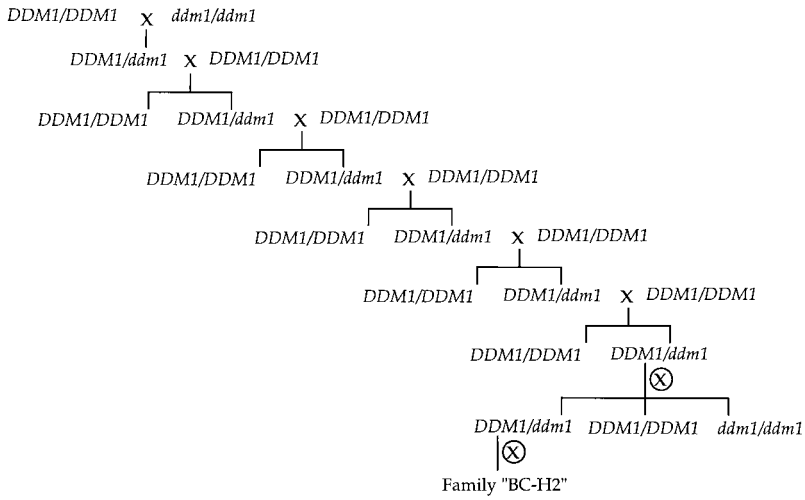


Figure 1.—Lineage of BC-H2 (progeny of the repeatedly backcrossed heterozygote).

gotes (*DDM1*/*ddm1*) produced by one outcross to wild-type plants contain 5mC in *Taq*I sites in amounts intermediate between those in the two parents; and (ii) repeated backcrossing of the heterozygotes to wild-type parents generates plants that contain an amount of 5mC at *Taq*I sites that approaches the amount found in wild-type plants. Given that 5mC levels of only *Taq*I sites were followed, however, it is possible that the wild-type *DDM1* allele is incompletely dominant over the examined *ddm1* mutant allele. More importantly, the extent of remethylation of genomic sequences hypomethylated by *ddm1* mutation has only been examined in *DDM1*/*ddm1* heterozygotes, not in *DDM1*/*DDM1* wild-type backgrounds.

In this article, we extend the previous studies of *Taq*I sites to the whole genome using HPLC and examine methylation of specific loci and alleles in the outcross progeny including *DDM1*/*DDM1* homozygotes by Southern analysis. The results substantiate our previous proposal and show that *ddm1*-induced hypomethylation in the majority of sequences in the Arabidopsis genome, both repeated and single-copy sequences, can be stably inherited both mitotically and meiotically. The stable transmission of DNA hypomethylation correlates with the stable property of the *ddm1*-induced developmental abnormalities.

MATERIALS AND METHODS

Plants and growth conditions: Isolation of *ddm1* mutants of *Arabidopsis thaliana* was reported by Vongs *et al.* (1993). The *ddm1-2* allele was used throughout. Plants were grown in a controlled environmental chamber under a long-day condition as described (Kakutani *et al.* 1996).

Measurement of 5mC content by HPLC: The levels of 5mC were measured by a modification of the reversed-phase HPLC method described by Kuo *et al.* (1980). Arabidopsis genomic DNA was isolated as described (Kakutani *et al.* 1995). To remove RNA, the DNA solution was treated with ribonuclease A, precipitated by adding an equal volume of 13% PEG, 1.6 m NaCl, and centrifuged at 27,400 × *g* for 2 min at 4°. The precipitate was rinsed with 70% ethanol, air dried, and dissolved in TE (pH 8). The solution was extracted twice with an equal volume of phenol/chloroform and once with chloroform. After addition of 1/4 volume of 10 m ammonium acetate (pH 7.7) and 2 volumes of ethanol, the DNA was precipitated by centrifugation at 7790 × *g* for 3 min at room temperature. The precipitate was washed with 70% ethanol, air dried, and dissolved in TE. The digestion of DNA was performed as described by Kuo *et al.* (1980). Digested samples were filtered [UFC4 TGC, pore size 10,000 (Millipore, Bedford, MA)] and resolved on a Purasil C_{18} column (4.6 × 150 mm; Waters Associates, Inc., Milford, MA) with a 60-min isocratic gradient of 10 mm ammonium phosphate buffer with 2.5% methanol (pH 5.6). The position of each nucleoside was determined using commercially available standards (Sigma, St. Louis). The values were calculated by integration of peak areas of absorbance at 280 nm with DataModule, Waters 741. The 5mC content ([5mC])/([5mC]+[C]) was normalized for absorbance difference between cytosine and 5mC.

Southern analysis of genomic DNA: Southern analysis of genomic DNA was performed as described by Ausubel *et al.* (1987) using the high-SDS hybridization buffer of Church and Gilbert (1984). Radiolabeled probes of 180-bp centromere repeat, rDNA repeat (Vongs *et al.* 1993), retrotransposon Ta3 (Konieczny *et al.* 1991), and m105 (Pruitt and Meyerowitz 1986) were generated using the Megaprime DNA-labeling system (Amersham, Arlington Heights, IL).

Repeated backcrossing: Figure 1 shows the lineage of the repeatedly backcrossed plants. *DDM1*/*DDM1* and *DDM1*/*ddm1* plants should segregate from the cross *DDM1*/*ddm1* × *DDM1*/*DDM1*. Plants from such a segregating family were used as material for the next backcrossing without determining the genotype. *DDM1*/*ddm1* plants were later identified by progeny tests, and progeny of the cross *DDM1*/*ddm1* × *DDM1*/*DDM1* was used for the next backcrossing generation.

RESULTS

Cytosine methylation levels in F_1 plants from a cross between a *ddm1* mutant and wild-type plants are intermediate between those of the parents: We have previously shown that heterozygotes (*DDM1*/*ddm1*) produced by crossing a *ddm1*/*ddm1* homozygote to a wild-type plant contain 5mC at *Taq*I sites (TCGA) in amounts halfway between those of the two parents (Vongs *et al.* 1993).

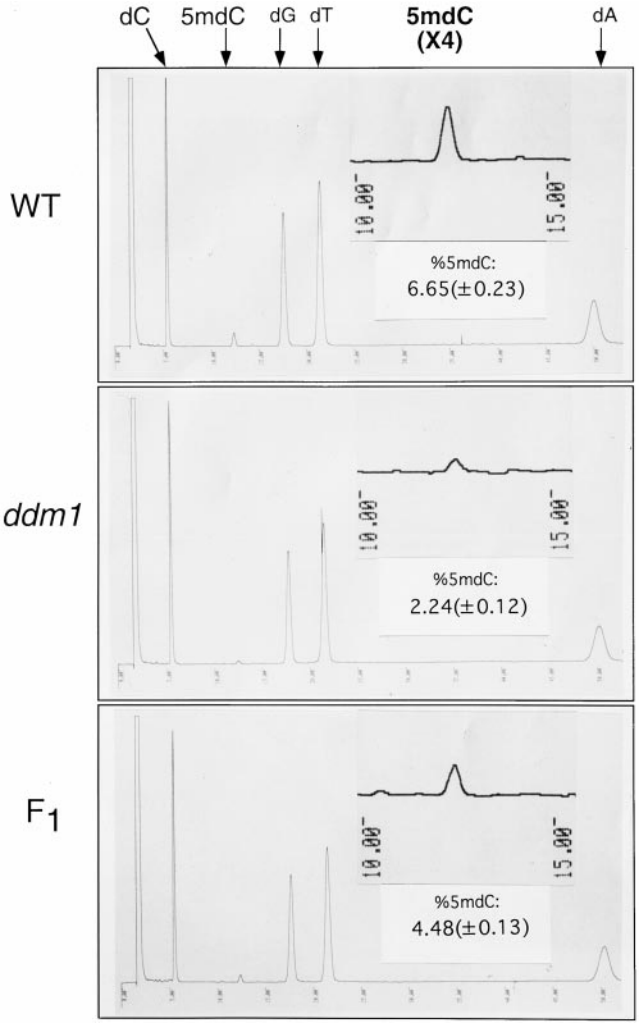


Figure 2.—5-methylcytosine (5mC) level in wild-type Columbia (WT), homozygous *ddm1* mutant, and the F_1 measured by reversed-phase HPLC. The values under the magnified chart represent the averages and deviations of four individual runs.

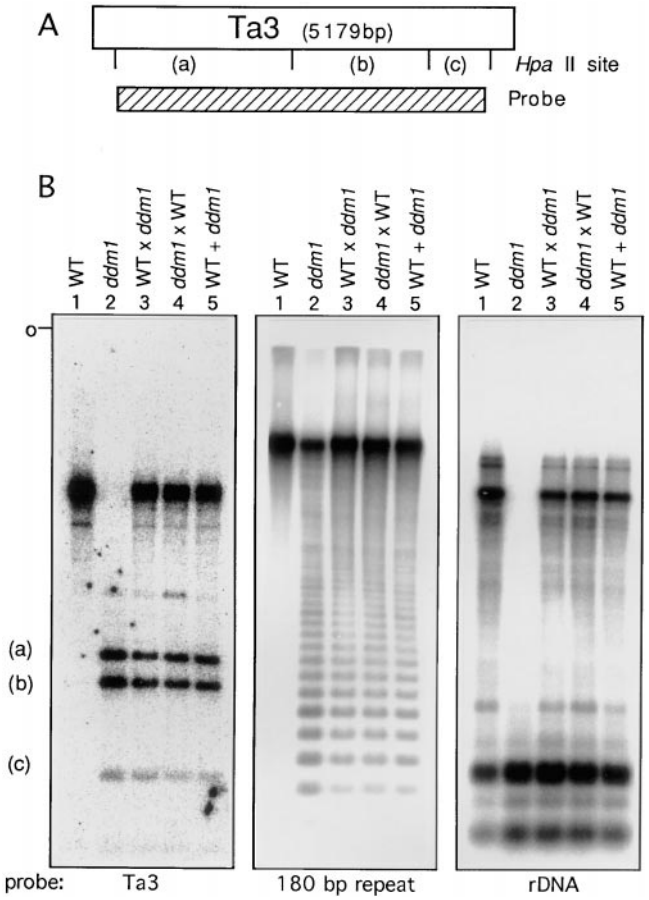


Figure 3.—Southern analysis of DNA methylation patterns of *Hpa*II sites in *ddm1* mutant, wild-type, and the F_1 plants. (A) *Hpa*II sites in Ta3 sequence. (B) The filter was probed with Ta3 (left), 180-bp centromere repeat (center), or rDNA (right). In each part, genomic DNA from the following plants was used: lane 1, wild-type Columbia (*DDM1*/*DDM1*); lane 2, *ddm1*/*ddm1*; lane 3, *DDM1*/*DDM1* × *ddm1*/*ddm1*; lane 4, *ddm1*/*ddm1* × *DDM1*/*DDM1*; lane 5, 1:1 mixture of *DDM1*/*DDM1* and *ddm1*/*ddm1* DNA.

Methylation in other sites, however, has not been examined. Here, we examined the 5mC content of the total genome as examined by HPLC analysis, which allows cytosine methylation at every site to be sampled. The 5mC content of the *ddm1* mutant genome was reduced to ~30% of the wild-type level (Figure 2), a value consistent with previous reports (Vongs *et al.* 1993; Ronemus *et al.* 1996). The F_1 heterozygotes (*DDM1*/*ddm1*), produced by crossing a *ddm1* homozygote to a wild-type plant, contain 5mC at levels halfway between those of the two parents (Figure 2), consistent with our previous study. At first glance, these results seem to suggest that the *ddm1* mutation is semidominant. However, our previous observations (Vongs *et al.* 1993) and the findings described below lead us to believe that this is not the case.

Although the results of TLC and HPLC show the overall amount of 5mC in the genome, the distribution of the genomic 5mC in different loci and different alleles cannot be analyzed by these methods. This was investigated by Southern analysis using a methylation-sensitive restriction endonuclease. Figure 3 shows the results of Southern analysis using the methylation-sensitive restriction enzyme, *Hpa*II, in the genome of *ddm1*/*ddm1* mutant, wild-type, and the F_1 plants. We examined three sequences: retrotransposon Ta3 (Konieczny *et al.* 1991), rDNA, and 180-bp centromere repeats (Vongs *et al.* 1993). Figure 3A illustrates the restriction map of Ta3. All four *Hpa*II sites are demethylated in the *ddm1* mutant, but methylated in wild-type plants. In DNA from the F_1 plants, both the top band and the three bottom bands were observed, indicating that *Hpa*II sites in the Ta3 locus are completely methylated in about half of the DNA, while all the *Hpa*II sites are unmethylated in the rest. Consistent with this interpretation, a mixture of genomic DNA from *DDM1*/*DDM1* and *ddm1*/*ddm1* plants gave essentially the same banding pattern as that

from a *DDM1*/*ddm1* plant (Figure 3B). F_1 plants from the cross *DDM1*/*DDM1* × *ddm1*/*ddm1* and the reciprocal cross gave the same banding pattern.

Similar results were obtained using two repeated sequences, 180-bp repeats and rDNA, as hybridization probes. These probes recognize tandemly repeated sequences clustering in two (rDNA) or five (180-bp repeats) unlinked loci of the *A. thaliana* genome. Both types of repeats are hypomethylated in *ddm1* mutants (Vongs *et al.* 1993). *Hpa*II sites in the rDNA sequences are completely demethylated in the *ddm1* mutants, whereas most of the sites are methylated in the wild type. *Hpa*II sites in the 180-bp repeats are demethylated in *ddm1* mutants but completely methylated in the wild type. For both of these repeated sequences, the extent of the methylation of F_1 appears to be intermediate between that of the two parents (Figure 3B). For the 180-bp probe, the ladder of bands did not shift upward, although the overall intensity was reduced. The absence of the shift in relative band intensities again suggests that about half of the DNA was hypomethylated as in the *ddm1* mutant, and the rest was normally methylated as in the wild-type parent.

Methylation status was determined not only by *DDM1* genotype but also by methylation status of the chromosome in the previous generation: To explain why the methylation level of DNA in the F_1 plants was intermediate between that of the two parents, three models were considered.

Model 1: The *ddm1* mutation is semidominant and causes incomplete genome methylation in heterozygotes (*DDM1*/*ddm1*).

Model 2: When the genotype of a haploid gametophyte is *ddm1*, the mutation results in hypomethylated chromosomes, which remain hypomethylated after fertilization and during the development of the next sporophyte generation.

Model 3: Hypomethylated chromosome segments originating from a *ddm1* mutant plant remain hypomethylated during meiosis and mitosis, resulting in hypomethylation of half of the chromosomes in F_1.

To distinguish among these three models, methylation of 180-bp repeats was examined in the progeny resulting from backcrosses of F_1 *DDM1*/*ddm1* to *DDM1*/*DDM1*. If model 1 or model 2 is correct, methylation of each progeny plant should be determined only by the genotype of the *DDM1* locus, plants with *DDM1*/*DDM1* should have normally methylated chromosomes, and *DDM1*/*ddm1* plants should have hypomethylated chromosomes. If model 3 is correct, most progeny should inherit hypomethylated 180-bp repeats (theoretically, $1 - (\frac{1}{2})^5 = \frac{31}{32}$, because 180-bp repeats on five centromeres should segregate). All of the examined progeny from a F_1 *DDM1*/*ddm1* × *DDM1*/*DDM1* cross ($n = 7$) and a reciprocal *DDM1*/*DDM1* × F_1 *DDM1*/*ddm1* cross ($n = 7$) had the hypomethylated ladder of centromere repeat bands, although the intensity of the ladder differed from plant to plant (Figure 4). These results are consistent with model 3, but neither model 1 nor 2 can explain the results (possibility that all of the 14 plants are *DDM1*/*ddm1*, 2^{-14}). Similarly, all 43 selfed F_2 progeny from a F_1 *DDM1*/*ddm1* plant showed a hypomethylated ladder of bands (Figure 5), confirming the conclusion that the methylation status was not determined by the *DDM1* genotype alone (possibility that none of the 43 F_2 plants is *DDM1*/*DDM1*, 0.75^{-43}). These results indicate that neither incomplete dominance (model 1) nor the effect of *ddm1* mutant allele on the gamete (model 2) can explain the hypomethylated chromosomes in F_1 plants and the progeny, whereas model 3 can explain all the results obtained.

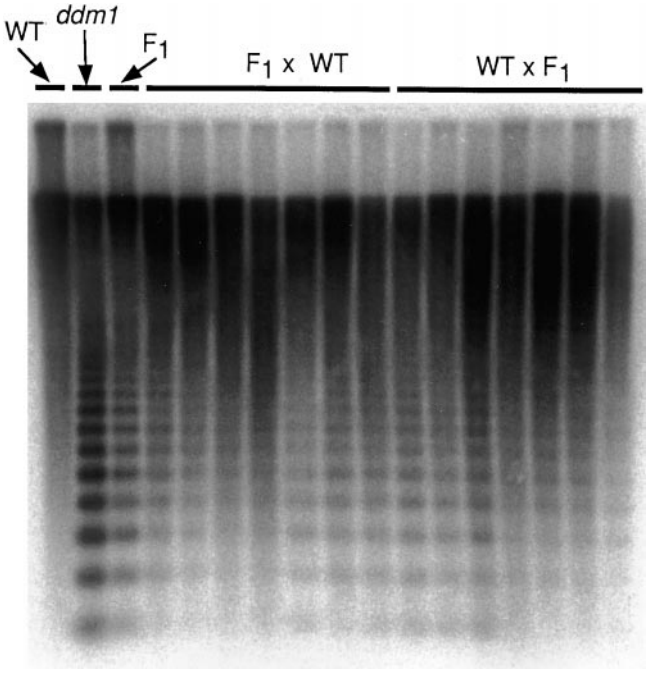


Figure 4.—Southern analysis of DNA methylation patterns of the 180-bp repeat in wild-type (*DDM1*/*DDM1*), *ddm1*/*ddm1*, F_1 (*DDM1*/*DDM1* × *ddm1*/*ddm1*), and outcross progeny of the F_1: (*DDM1*/*DDM1* × *ddm1*/*ddm1*) × *DDM1*/*DDM1* and *DDM1*/*DDM1* × (*DDM1*/*DDM1* × *ddm1*/*ddm1*).

Is the *ddm1* mutation completely recessive? It is possible, however, that more than one mechanism is responsible for the hypomethylated chromosomes in F_1 plants and their progeny. For example, inefficient *de novo* methylation and incomplete dominance of the *DDM1* allele over the *ddm1* allele together may result in hypomethylated chromosomes in *DDM1* backgrounds. To examine whether *ddm1* is completely recessive, in other words, whether a *DDM1*/*ddm1* heterozygote plant can methylate genomic cytosine as efficiently as a *DDM1*/*DDM1* homozygote, heterozygotes created by repeated backcrossing were used. Figure 1 illustrates the lineage of the materials used. We have previously suggested that the *ddm1* mutations are recessive because repeated backcrossing of heterozygotes to wild-type parents gen-

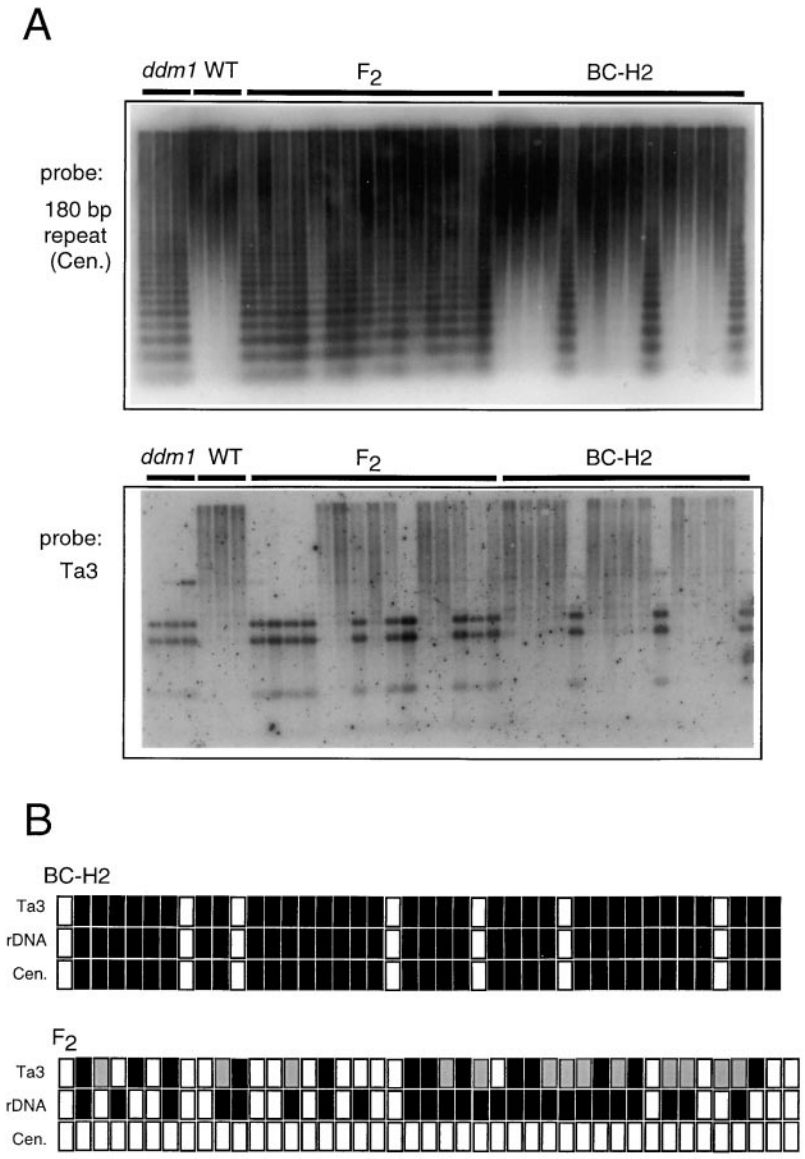


Figure 5.—(A) Southern analysis of the DNA methylation pattern in the F_2 family (from a cross *ddm1*/*ddm1* × *DDM1*/*DDM1*) and BC-H2 family (progeny from a backcrossed *DDM1*/*ddm1*, see the lineage in Figure 1) using *Hpa*II. (B) Summary of the methylation status of three genomic sequences in F_2 and BC-H2. Generally, black and white boxes represent normal methylation and hypomethylation in the *Hpa*II sites of the sequence examined, respectively. For Ta3, a gray box indicates one copy methylated and the other copy hypomethylated. For rDNA, a white box indicates no top band, in other words, all *Hpa*II sites are unmethylated. A black box indicates that *Hpa*II sites are methylated in at least one of the rDNA clusters. For 180-bp repeats (Cen.), a white box means a ladder of bands was observed, indicating that one or more copies of the repeats were hypomethylated in the *Hpa*II sites. A black box means that no ladder was observed.

erates plants that contain amounts of 5mC in *Taq*I sites that approach the amount found in wild-type plants (Vongs *et al.* 1993). To see how complete the dominance of *DDM1* allele over *ddm1* allele is, the methylation of specific genomic sequences in the progeny from such a backcrossed *DDM1*/*ddm1* was examined. Figure 5A shows the results obtained. In contrast to the F_2 family (progeny of a heterozygote *DDM1*/*ddm1* without backcrossing) in which all the plants had hypomethylated 180-bp repeats, only about one-quarter of the progeny from the backcrossed heterozygote (BC-H2) show the ladder of hypomethylated 180-bp repeats. Figure 5B summarizes the methylation status of the three genomic sequences in the F_2 and the progeny of a backcrossed heterozygote. About one-quarter of the progeny of both types had hypomethylation in all of the sequences examined (*i.e.*, 180-bp repeats, rDNA, and Ta3), suggesting that these individuals are *ddm1*/*ddm1*. The wild-type methylation pattern of the remaining three-quarters of the progeny of the backcrossed heterozygote indicates that *DDM1*/*ddm1* plants are indistinguishable from *DDM1*/*DDM1* plants in their ability to methylate all the sequences examined. The backcrossed heterozygote parent contained fully methylated chromosomes due to dilution of the hypomethylated chromosome by the normally methylated chromosomes during the repeated backcrossing. Furthermore, these results suggest that model 2 is not correct. The lack of detectable hypomethylation in *DDM1*/*ddm1* plants demonstrates that hypomethylation does not occur in *ddm1* gametophytes. The presence of hypomethylated chromosomes in all the F_2 progeny indicates that hypomethylated chromosome segments can be inherited independently of the *ddm1* mutation.

Stable inheritance of hypomethylation of rDNA and a retroelement in *DDM1*/*DDM1* background: The results shown in the previous sections indicate that one copy of wild-type *DDM1* allele is sufficient for normal *DDM1*

function. Therefore, the observation that the methylation level of F_1 is precisely intermediate between that of the two parents suggests that the rate of *de novo* methylation of unmethylated chromosome segments from a *ddm1* mutant parent is extremely slow even in the wild-type *DDM1* backgrounds. To test this interpretation, we estimate the rate of *de novo* methylation of hypomethylated sequences in *DDM1*/*DDM1* background by Southern analysis. As hypomethylated sequences remain hypomethylated even when segregated from the potentiating *ddm1* mutation, we could generate *DDM1*/*DDM1* plants with unmethylated Ta3 or rDNA sequences from progeny of a cross between *ddm1* mutants and wild-type plants.

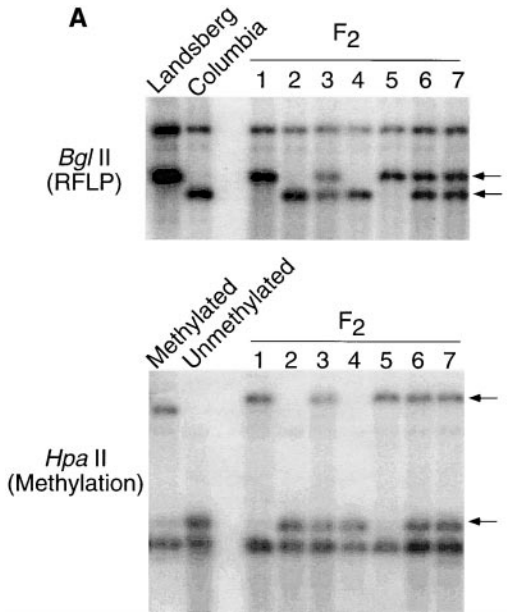
We first investigated selfed progeny of a *DDM1*/*DDM1* plant homozygous for hypomethylated nucleolus organizer regions (NORs) on chromosomes 2 and 4. From 12 F_2 progeny of a cross: *ddm1*/*ddm1* × *DDM1*/*DDM1*, 1 plant (95-89/10) with normally methylated Ta3 and partially hypomethylated rDNA was selected. Among 24 selfed progeny of 95-89/10, 5 plants had hypomethylated Ta3, indicating that 95-89/10 is *DDM1*/*ddm1*. All four copies of the rDNA loci (*i.e.*, two copies each of nucleolus organizer regions NOR2 and NOR4) were hypomethylated in 7 plants of this family. One of these plants was determined to be *DDM1*/*DDM1* by progeny tests. Forty-five progeny were examined in this family and no detectable remethylation of the rDNA sequences was detected, demonstrating stable inheritance of hypomethylation in a large number of rDNA repeat sequences (data not shown). All 45 progeny had normally methylated Ta3 sequences, confirming that the parent was *DDM1*/*DDM1*.

Similarly, hypomethylated Ta3 remained hypomethylated in *DDM1*/*DDM1* background. From 12 F_2 progeny of a cross *ddm1*/*ddm1* × *DDM1*/*DDM1*, 4 plants with methylated rDNA and heterozygous for Ta3 methylation alleles (one copy of Ta3 was normally methylated and the other copy hypomethylated) were identified. One of them (95-89/6) was determined to be *DDM1*/*DDM1* by progeny tests. The methylation status of the Ta3 locus was determined in 47 progeny of 95-89/6. Among the progeny, 11 plants were homozygous for the methylated Ta3 alleles, 27 plants were heterozygous, and 10 plants were homozygous for the hypomethylated Ta3 allele. Three plants homozygous for hypomethylated Ta3 alleles were used here to examine *de novo* methylation of hypomethylated Ta3 in a *DDM1*/*DDM1* background. In all the examined progeny from these 3 plants ($n = 24 + 24 + 23$), the four *Hpa*II sites (see Figure 3A) remained hypomethylated (data not shown). In conclusion, hypomethylation of Ta3 and rDNA induced by the *ddm1* mutation was stably inherited even in *DDM1*/*DDM1* background.

Stable inheritance of hypomethylation slowly induced by *ddm1* mutation: We have previously shown that *ddm1* mutation induces a variety of developmental abnormalities by causing heritable changes on unlinked loci (Kakutani *et al.* 1996; Kakutani 1997). As this induction does not seem to be a random mutation event, we have proposed that it is due to *ddm1*-induced epigenetic change in other loci (Kakutani 1997). Consistent with this interpretation, we found slowly accumulating hypomethylation in some of the single-copy sequences (Kakutani *et al.* 1996), such as m105 and m118 (Pruitt and Meyerowitz 1986). Most of the repeated sequences methylated in wild-type *A. thaliana* are hypomethylated in *ddm1* mutants recovered in the segregating population. In contrast, although some of single-copy sequences such as Ta3 and telomere-associated sequence YpAtT1 are hypomethylated immediately as repeated sequences, most of the single-copy sequences are unaffected (Vongs *et al.* 1993; Ronemus *et al.* 1996). These unaffected single-copy sequences generally become hypomethylated during the propagation by repeated selfing. To see if such slowly induced hypomethylation is also meiotically heritable, DNA of an F_2 family from an interstrain cross between a plant with two hypomethylated m105 alleles (strain Columbia) and a wild-type plant (Landsberg) was analyzed. The origin of m105 allele could be detected by examining a *Bgl*II RFLP between the Landsberg and Columbia strain (Chang *et al.* 1988), as shown in the top of Figure 6A. The methylation status of the m105 sequence was detected using the methylation-sensitive restriction enzyme *Hpa*II, as shown in the bottom of Figure 6A. Homozygous *ddm1* mutants in each class were identified by hybridizing the filter with a cloned *A. thaliana* rDNA sequence (in parentheses in Figure 6B). The rDNA sequence becomes hypomethylated in *ddm1* mutants before repeated selfing (Vongs *et al.* 1993) and can be used for identifying *ddm1* homozygotes immediately. As shown in Figure 6B, all of the 79 hypomethylated Columbia m105 alleles (38 from 19 Columbia m105 homozygotes and 41 from heterozygotes) remain hypomethylated through an outcross and a selfing. Out of these 79 hypomethylated alleles, 57 (30 from 15 Columbia m105 homozygotes and 27 from heterozygotes) were in a *DDM1*/− background. One demethylation event was observed in 1 of the 20 plants homozygous for the Landsberg m105 allele (Figure 6B). As this plant was *ddm1*/*ddm1*, the demethylation event is consistent with our previous observation that slow and stochastic hypomethylation of the m105 sequence occurs in *ddm1* mutant backgrounds. In conclusion, hypomethylation of the m105 sequence was meiotically transmitted even upon segregation from the potentiating *ddm1* mutation.

DISCUSSION

The results presented here indicate that (i) *DDM1* gene function is not necessary during the gametophyte stage, (ii) the *ddm1-2* mutation is completely recessive, and (iii) remethylation of sequences hypomethylated



B

Origin of m105	METHYLATION −	±	+
Col	19 (4)	0	0
Het	0	41 (14)	0
La	0	1 (1)	19 (2)

Figure 6.—Stable inheritance of hypomethylation of the m105 locus. DNA were prepared from F_2 plants from an interstrain cross between a wild-type plant (Landsberg) and a *ddm1* mutant plant (Columbia, line#6) with hypomethylation of the m105 locus. After Southern blotting, the membrane was probed with a 3.3-kb *Eco*RI subclone from the single-copy clone m105 (Pruitt and Meyerowitz 1986). (A) Top: The origin of the m105 allele was detected by RFLP (Chang *et al.* 1988) after *Bgl*II cleavage. *DDM1* and *ddm1* plants in Columbia background showed the same band pattern (data not shown). Bottom: The methylation status of the m105 locus was detected by *Hpa*II cleavage. The hypomethylation of the m105 allele had been generated during the self-pollination of the *ddm1* mutant (Kakutani *et al.* 1996) as shown in the two left lanes in the bottom. Methylated, *ddm1* mutant plant before repeated selfing; Unmethylated, *ddm1* mutant plant after six times of selfing. Both of them were in Columbia background; but *DDM1* plants in Landsberg background also showed the top band (data not shown). (B) Methylation of m105 alleles in the F_2 population. Number of plants is shown, with numbers of *ddm1* plants in parentheses. Origin of m105: Col, Columbia homozygotes; Het, heterozygotes; La, Landsberg homozygotes. Methylation: −, signal at 2.1-kb position as plants 2 and 4; ±, signal at both 2.1 kb and 4.7 kb as plants 3, 6, and 7; +, signal at 4.7 kb as plants 1 and 5 of the F_2.

by the *ddm1-2* mutation occurs extremely slowly, if at all, in wild-type *DDM1* backgrounds.

A few rounds of DNA replication occur during the gametophyte stage of development: three for the female gametophyte to make egg cells and two for the male gametophyte to make sperm nuclei. If the maintenance methylation machinery does not function in a haploid *ddm1* gamete, a substantial loss of DNA methylation should result, but this is not the case. One possible interpretation for the dispensability of *DDM1* gene function in the gametes is that the function is developmental-stage-specific and not required in the gametophyte stage. An alternative interpretation is that sporophytic *DDM1* gene product remaining in the gametes is sufficient for the normal *DDM1* function.

A more important conclusion from the results presented here is that *ddm1*-induced hypomethylation in the majority of sequences in the Arabidopsis genome, both repeated and single-copy sequences, can be stably inherited through both mitotic and meiotic cell divisions. This indicates that epigenetic information, in the form of differential DNA methylation, can be transmitted between plant generations. Transgenic Arabidopsis plants expressing the *MET1* gene in an antisense orientation (*MET1*as) exhibit a reduction in genomic methylation (Finnegan *et al.* 1996; Ronemus *et al.* 1996). In progeny of the transgenic plants, hypomethylation of the 180-bp repeats is transmitted even to the plants losing the transgene (Finnegan *et al.* 1996; Ronemus *et al.* 1996). The transmission was, however, not fully penetrant, and remethylation of at least the 180-bp repeats occasionally occurs in both outcross progeny (Ronemus *et al.* 1996) and selfed progeny from hemizygotes (J. Finnegan, personal communication) that do not inherit the transgene. Thus, DNA remethylation efficiency may differ between *ddm1* and *MET1*as plants. Similarly, developmental abnormalities induced by *MET1* antisense expression are often unstable compared to those induced by *ddm1* mutation. For example, phenotypic revertants were occasionally found among outcross progeny of late-flowering *MET1*as plants without the transgene (Ronemus *et al.* 1996), in contrast to the stable inheritance of late-flowering traits in outcross progeny from *ddm1* mutant (Kakutani 1997).

The basis for these observed differences in stability is not clear. It may reflect a difference in the distribution of the hypomethylated sequences and the extent to which the sequences are hypomethylated. In *ddm1* mutants, repeated sequences are more effectively hypomethylated than single-copy sequences (Vongs *et al.* 1993), while both single-copy and repeated sequences are hypomethylated in *MET1*as lines (Ronemus *et al.* 1996). If there were positive cooperativity in *de novo* methylation of the endogenous genes, the extent of hypomethylation of particular genomic regions would affect the remethylation efficiency.

Alternatively, the effect of *ddm1* mutation might be qualitatively different from that of *MET1*as. The *DDM1* gene product is not likely to be DNA methyltransferase, because nuclear extracts of the *ddm1* mutant have as much DNA methyltransferase activity as those of the wild type, and the *ddm1* gene does not map to any known methyltransferase structural gene (Kakutani *et al.* 1995). It is possible that the hypomethylation is a

secondary effect of *ddm1* mutation, and the primary effect is on another epigenetic state such as chromatin structure. The primary effect of *ddm1* mutation could be more stably inherited than the hypomethylation itself. Meiotically stable inheritance of the epigenetic chromatin state has been found in fission yeast for both the centromere (Ekwall *et al.* 1997) and mating-type locus (Grewal and Klar 1996), despite a lack of detectable DNA methylation in its genome (Antequera *et al.* 1984).

Mutation of *ddm1* has recently been found to release silencing of repeated hygromycin phosphotransferase transgenes driven by the 35S promoter (Mittelsten-Scheid *et al.* 1998), repeated *CHS* transgenes (Furner *et al.* 1998), and the endogenous *PAI2* gene (Jeddeloh *et al.* 1998). Molecular and genetic characterization of *ddm1*, *MET1*as, and other recently identified Arabidopsis mutants affecting gene silencing (Dehio and Schell 1994; Furner *et al.* 1998; Mittelsten-Scheid *et al.* 1998) would be useful for further understanding the basis for the inheritance of epigenetic states in plant genes.

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