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# Developmental abnormalities and epimutations associated with DNA hypomethylation mutations

(epigenetic/DNA methylation mutant/*Arabidopsis thaliana*/*ddm1*)

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**ABSTRACT** **A number of aberrant morphological phenotypes were noted during propagation of the *Arabidopsis thaliana* DNA hypomethylation mutant, *ddm1*, by repeated self-pollination. Onset of a spectrum of morphological abnormalities, including defects in leaf structure, flowering time, and flower structure, was strictly associated with the *ddm1* mutations. The morphological phenotypes arose at a high frequency in selfed *ddm1* mutant lines and some phenotypes became progressively more severe in advancing generations. The transmission of two common morphological trait syndromes in genetic crosses demonstrated that the phenotypes are caused by heritable lesions that develop in *ddm1* mutant backgrounds. Loss of cytosine methylation in specific genomic sequences during the selfing regime was noted in the *ddm1* mutants. Potential mechanisms for formation of the lesions underlying the morphological abnormalities are discussed.**

DNA modification has been postulated to play a central role in epigenetic regulation by modulating access to the genetic information (1, 2). Much research has centered on the biological significance of the post-replicative addition of a methyl group to the cytosine ring, a DNA modification widespread in both prokaryotes and eukaryotes. The importance of DNA methylation in regulation of a number of cellular processes in prokaryotes, including restriction-modification, transposition, DNA repair, and transcription, is well established (3).

The function of cytosine methylation in eukaryotic cells is less clear. A large body of experimental evidence points to a role for DNA methylation in modulation of gene expression (4, 5). A correlation between increased cytosine methylation and transcriptional quiescence holds for many examples (6), suggesting a role for DNA methylation in maintenance of transcriptional inactivity. Methylation inhibitor (7) and mutant studies (8, 9) have supported the role of DNA methylation in propagation of established transcriptionally quiescent states. However, exceptions exist where increased cytosine methylation is positively correlated with gene activity (8, 10, 11). Moreover, proper gene expression can occur without the contribution of cytosine methylation in some organisms as demonstrated by the existence of eukaryotic species that lack detectable amounts of 5-methylcytosine (12–14).

DNA methylation mutants provide experimental systems to directly address the function of cytosine methylation without the problems associated with correlative studies or the use of methylation inhibitors. The embryo-lethality of the murine engineered DNA methyltransferase mutations clearly demonstrates that cytosine methylation is necessary for completion of early mouse development (15). Further studies with these engineered mutations showed that cytosine methylation is necessary for maintenance of parental imprinting (8) and proper regulation of the X-chromosome inactivation control gene, *Xist* (9). DNA hypomethylation mutants have also been isolated in the filamentous fungus *Neurospora* (16). In contrast to the mouse DNA methyltransferase mutants, *Neurospora* mutants that lack detectable 5-methylcytosine exhibit only mild and variable morphological defects. The different phenotypic consequences of DNA hypomethylation mutations in different organisms point to the diversity with which eukaryotes use DNA modification and the need for parallel studies of DNA methylation in different model systems.

We have been pursuing a genetic approach to an understanding of the function and regulation of eukaryotic DNA methylation through the study of *Arabidopsis thaliana* mutants with altered cytosine methylation. We have previously described the isolation of two independent recessive alleles of the *DDM1* (decrease in DNA methylation) locus that cause an approximately 70% reduction in genomic 5-methylcytosine content (17). The *ddm1* mutations do not map to known cytosine methyltransferase genes (18), nor do they affect cytosine methyltransferase activity detectable in nuclear extracts or metabolism of the methyl group donor, *S*-adenosylmethionine (19). Apparently, the *ddm1* mutations disrupt a novel component of the methylation machinery or affect the methylation of genomic sequences by an indirect mechanism.

Homozygous *ddm1* mutants display only weak morphological changes when first identified in segregating populations (19). Despite the lack of dramatic immediate phenotypes, *A. thaliana ddm1* mutations are associated with the delayed onset of a number of severe developmental abnormalities as described in this report. Our genetic analyses indicate that *ddm1* mutations lead to the formation of heritable lesions at unlinked loci that, in turn, cause the morphological phenotypes.

## MATERIALS AND METHODS

**Plant Growth.** Plants were grown on standard soil mixtures [60% Scotts Redi-earth/40% vermiculite; or 62.5% SupermixA (Sakata, Yokohama, Japan)/25% vermiculite/12.5% perlite) in a greenhouse or in an environmental chamber at 20–25°C under 16–24 hr of illumination per day, with the exception of the clam mapping population, which was grown on axenic solid media (20).

**Genetic Mapping.** All loci were mapped relative to strain-specific molecular markers (21, 22) in segregating $F_2$ families. The *BAL* mapping families were generated by a parental inter-strain cross (severe ball *DDM1*/*ddm1-2* Columbia X wild-type Landsberg erecta strain) that yielded aphenotypic $F_1$ plants. Four *DDM1*/*DDM1* $F_1$ plants, identified by progeny testing, were selfed to generate $F_2$ mapping populations. The lesion(s) at the *BAL* locus acted in a recessive manner in the inter-strain crosses and the $F_2$ mapping populations segregated 128 normal: 40 severe ball plants (null hypothesis, $H_o$ = 3:1 :: normal: severe ball; $\chi^2$ = 0.13; $P \approx 0.7$). We prepared genomic

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DNA from 26 severe ball $F_2$ plants and determined genotypes for distributed PCR-based molecular markers to find tight linkage between the lesion at the *BAL* locus and the *AG* marker (22) (2 recombinant chromatids of 52 total). The map assignment was confirmed by demonstration of linkage to another chromosome 4 marker, *PG11* (22) (6 recombinant chromatids of 52 total). Consideration of the *AG* genotypes of the six chromatids showing recombination between *PG11* and *BAL* indicated that the *BAL* locus maps centromere-proximal to *AG*.

The *CLM* mapping families were generated by a parental inter-strain cross (severe clam *ddm1-1/ddm1-1* Columbia X wild-type Landsberg erecta strain) that yielded aphenotypic *DDM1/ddm1-1* $F_1$ plants. These $F_1$ individuals were selfed to generate $F_2$ mapping populations [271 normal: 71 clam plants ($H_o$ = 3:1 :: normal: clam; $\chi^2 = 3.3$; $0.1 > P > 0.05$)]. We prepared genomic DNA from 48 clam $F_2$ plants and determined genotypes for distributed molecular markers to find linkage to the lesion underlying the clam phenotype. The clam locus (*CLM*) is located in the center of the interval between *GL1* and *BGL1* markers (22) (12 recombinant chromatids of 96 total for each marker).

Using a separate $F_2$ Columbia/Landsberg mapping population, we mapped the *ddm1-2* mutation to a position approximately 15 centimorgans distal to the *LFY* marker (22) on chromosome 5.

**Measurement of Global 5-Methylcytosine Content.** The methylation of cytosine in genomic 5′-TCGA-3′ sites was determined by the thin-layer chromatography method of Cedar *et al.* (23), as modified by Vongs *et al.* (17). Total genomic 5-methylcytosine content was determined by digestion of total genomic DNA to nucleotides followed by separation and quantitation by high-performance liquid chromatography (24).

**Restriction Digestion and Southern Blot Analysis.** *A. thaliana* DNA was prepared using the urea lysis method (25). Restriction digestions were performed using the manufacturers' (New England Biolabs and Boehringer Mannheim) specifications. The digested genomic DNA samples were size-fractionated by agarose gel electrophoresis and transferred to Hybond N+ (Amersham) nylon membranes. The membranes were hybridized with radiolabeled probes prepared by the random priming method (Multiprime system, Amersham) following the high SDS hybridization method of Church and Gilbert (26). The filters were washed at 65°C in 0.2× SSC/0.1% SDS, and the hybridization signals were detected by digital image analysis (FUJIX BAS 2000).

## RESULTS

***Arabidopsis ddm1* Mutations Are Associated with the Slow Onset of Severe Developmental Abnormalities.** We noted a high incidence of morphological abnormalities in *ddm1* homozygous lines propagated by repeated self-pollination (Fig. 1 and Table 1). The onset of the abnormalities was strictly associated with the *ddm1* mutations. The *ddm1/ddm1* lines were first backcrossed six times to recurrent parental lines to remove unlinked mutations. Similar morphological defects were conditioned by *ddm1* mutations in at least two genetic backgrounds, Columbia and Landsberg erecta (J.A.J., unpublished). Moreover, the severe developmental defects were seen in selfed lines carrying independently isolated *ddm1* alleles arguing against any contribution from additional mutations closely linked to *ddm1*. To control for the effects of inbreeding, we monitored the phenotypes of 14 *DDM1/DDM1* and *ddm1-2/ddm1-2* lines derived from a single segregating family and propagated by self-pollination in parallel. Plants with abnormal phenotypes were never seen in the *DDM1/DDM1* lines indicating that the onset of abnormal phenotypes was not caused by a non-specific inbreeding depression phenomenon operating in wild-type lines.

A spectrum of morphological abnormalities was seen in the 14 *ddm1/ddm1* mutant lines. Table 1 illustrates the morphological phenotypes scored after six generations of self-pollination. All of the *ddm1/ddm1* lines contained plants exhibiting aberrant morphologies, including reduction or increase in apical dominance, short internode length, late flow-

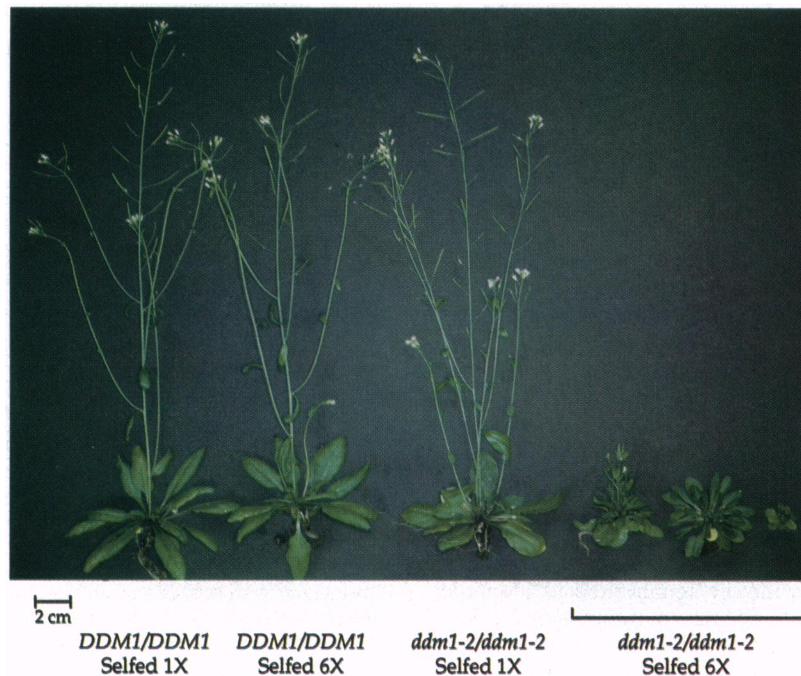


FIG. 1. Morphological phenotypes seen in *ddm1/ddm1* lines propagated by self-pollination. Severe abnormal morphological phenotypes were only seen in *ddm1* mutant lines and only occurred after propagation of the mutant lines by repeated self-pollination. Weak morphological abnormalities, such as slightly reduced apical dominance and rounder leaves (19), were evident in 1× selfed *ddm1* lines. Phenotypes differ among *ddm1* mutant lines (left to right: representatives of lines 1, 2, and 9). All plants were the same age (40 days) and were grown in parallel, under the same environmental conditions.

Table 1. Spectrum of morphological phenotypes and epimutations in 14 *ddm1-2/ddm1-2* and 14 *DDM1/DDM1* control lines after six generations of self-pollination

| | Genotype and line number | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ddm1-2/ddm1-2* | | | | | | | | | | | | | | *DDM1/DDM1* |
| Phenotype | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8* | 9 | 10 | 11 | 12 | 13 | 14 | 15–28 |
| Reduced apical dominance† | 5/5 | | | 3/6 | 6/6 | 2/6 | 1/5 | 6/6 | 3/6 | | | | | 1/6 | 0/84 |
| Short internode | 5/5 | | | | | 2/6 | | 6/6 | 1/6 | | | | | | 0/84 |
| Late flowering‡ | 5/5 | 5/5 | 1/6 | 2/6 | | 2/6 | | 3/6 | | 2/6 | 5/6 | | 1/6 | 1/6 | 0/84 |
| Small leaves§ | | | | | | | 1/5 | | 2/6 | | 1/6 | | 1/6 | 1/6 | 0/84 |
| Increased cauline leaf number¶ | | 3/5 | | 1/6 | | 1/6 | | | | 4/6 | 3/6 | | | | 0/84 |
| Reduced fertility | | | | 4/6 | | 1/6 | 3/5 | 6/6 | 3/6 | 1/6 | | 4/5 | 1/6 | 2/6 | 0/84 |
| Abnormal flowers‖ | | 2/5 | | 1/6 | | | | | | | 1/6 | 4/5 | | 2/6 | 0/84 |
| Epimutation 1 | 0/4 | 0/4 | 4/4 | 4/4 | 0/4 | 4/4 | 0/4 | 0/2 | 0/4 | 0/4 | 2/4 | 0/4 | 0/4 | 1/4 | 0/28 |
| Epimutation 2 | 4/4 | 3/4 | 0/4 | 0/4 | 2/4 | 0/4 | 0/4 | 1/2 | 2/4 | 2/4 | 0/4 | 0/4 | 2/4 | 0/4 | 0/28 |
| Epimutation 3 | 0/4 | 4/4 | 1/4 | 0/4 | 4/4 | 2/4 | 0/4 | 1/2 | 0/4 | 0/4 | 4/4 | 4/4 | 0/4 | 4/4 | 0/28 |

Fourteen *ddm1-2/ddm1-2* and fourteen *DDM1/DDM1* plants were identified in a segregating population generated by self-pollination of a *DDM1/ddm1-2* heterozygote (6× backcrossed). Independent lines were generated by self-pollination of each of these plants. In each generation, plants were randomly selected for phenotypic examination and seeds from self-pollination of a random individual plant were used as the source of the next generation. After six generations of self-pollination, approximately six individuals were selected at random and scored phenotypically. Plants displaying the morphological phenotypes are indicated by the first number in the fraction, whereas the total number of plants examined is given in the denominator. Blanks indicate that no abnormal phenotype was seen. In addition, we used Southern blot analysis (see Fig. 4) to score plants for the loss of methylation sites recognized by the genomic clones m105 (epimutations 1 and 2) and m118 (epimutation 3). The number of epimutations is indicated over the total number of alleles examined.

*Phenotypes from line 8 were scored after five self-pollinations; the line could not be propagated further because of sterility.

†Several flowering shoots (bolts) are produced simultaneously, in contrast to the dominant primary shoot seen in wild-type *A. thaliana*.

‡Plants flowered more than 15 days later than wild-type. The mean ± standard deviation of the flowering date for the 84 plants in the *DDM1/DDM1* (lines 15–28) = 32.3 ± 1.6 days.

§Rosette leaf length ≤ 20% of wild-type. The mean ± standard deviation of the length of the largest rosette leaf for the 84 plants in the *DDM1/DDM1* (lines 15–28) = 31.2 ± 4.1 mm.

¶More than 10 cauline leaves. The mean ± standard deviation of the cauline leaf number for the 84 plants in the *DDM1/DDM1* (lines 15–28) = 4.2 ± 0.8.

‖Reduced sepal number (lines 2, 4, and 11) or unfused carpels (lines 12 and 14).

ering, small leaf size, increased cauline leaf number, and reduced fertility. In addition, some lines displayed plants with abnormal flowers. Plants with reduced sepal number were noted in 3 of 14 *ddm1/ddm1* selfed lines and plants with hooked and partially unfused carpels were seen in two of the *ddm1/ddm1* selfed lines. After 7 generations of self-pollination, 5 of 14 *ddm1/ddm1* lines exhibited a high degree of sterility or seedling lethality (note that line 8 died out after five generations) (data not shown).

While there were differences in the spectrum of the phenotypes among the 14 *ddm1/ddm1* lines, some of the abnormal characters occurred together. One combination of phenotypes is characterized by an increase in apical dominance, an increase in cauline leaf number, and a delay in time to flowering (lines 2, 10, and 12 in Table 1). Another commonly seen combination of phenotypes, which we refer to as the "ball" syndrome, is characterized by reduced apical dominance, twisted leaves, and small plant size (Fig. 2). The severity of the ball syndrome was progressive with more pronounced phenotypes exhibited by plants in families resulting from higher numbers of self-pollinations (Fig. 2). Variability in the severity of the phenotype among siblings in advanced selfed generations was frequently noted.

**Independent Segregation of the Morphological Traits and the Potentiating *ddm1* Mutations.** To learn more about the basis of the phenotypes seen in the *ddm1/ddm1* selfed lines, we followed the inheritance of a subset of the phenotypes in genetic crosses. The ball syndrome is inherited as a simple Mendelian monogenic trait. Crosses between *ddm1/ddm1* phenotypic ball plants (strain Columbia) and wild-type Columbia plants yielded plants with normal phenotypes and intermediate ball phenotypes. $F_2$ generations derived by selfing the phenotypically intermediate plants contained plants with normal, intermediate, and severe ball phenotypes in a 1:2:1 ratio, respectively, suggesting the segregation of a semi-dominant lesion (Table 2). Inheritance of the ball phenotype in the $F_2$ generation was independent of the segregation of the *ddm1* mutation (Table 2). Starting with a *DDM1/ddm1-2* severe ball $F_2$ plant, we have generated several severe ball *DDM1/DDM1* lines in which no normal plants were seen through three generations of self-pollination (approximately 50 individuals examined per line per generation; data not shown).

The *ddm1* mutation and the locus responsible for the ball phenotype (*BAL*) were mapped relative to strain-specific genetic polymorphisms in segregating $F_2$ populations as de-

Table 2. Independent segregation of *ddm1* and a single locus controlling the ball phenotype

| Phenotype | Non-mutant *DDM1/_* | Mutant *ddm1-2/ddm1-2* | Totals |
|---|---|---|---|
| Normal | 23 | 7 | 30 |
| Intermediate ball | 45 | 19 | 64 |
| Severe ball | 18 | 10 | 28 |
| Totals | 86 | 36 | 122 |

A reciprocal cross between a phenotypic ball *ddm1-2/ddm1-2* plant (strain Columbia) and a wild-type Columbia plant resulted in $F_1$ *DDM1/ddm1-2* plants, some of which displayed an intermediate ball phenotype. The direction of the cross did not affect the results. Two independent intermediate ball $F_1$ plants were selfed to generate two segregating $F_2$ families. Pooled phenotype data from the $F_2$ families are shown. The ball phenotype was scored as severe (small rosette, twisted leaves, reduced apical dominance), intermediate (medium rosette size with ruffled leaf surface), or absent (normal wild-type). $F_2$ *ddm1/ddm1* individuals were identified by Southern blot analysis by monitoring the loss of *Hpa*II restriction endonuclease modification of genomic rDNA genes (17). The *ddm1-2* mutation segregates as a single recessive monogenic factor ($H_o$ = 3:1 :: non-mutant: mutant; $\chi^2 = 1.3$; $P \approx 0.25$). The ball phenotype was inherited as a semi-dominant monogenic trait in this intra-strain cross ($H_o$ = 1:2:1 :: normal: intermediate ball: severe ball; $\chi^2 = 0.36$ with df = 2; $P \approx 0.8$). The ball trait and the *ddm1-2* mutation segregated independently ($H_o$ = independent segregation; $\chi^2 = 1.1$ with df = 2; $P \approx 0.5$).

scribed in *Materials and Methods*. The *DDM1* locus maps to the distal portion of the lower arm of chromosome 5, whereas the *BAL* locus maps to the lower arm of chromosome 4 (Fig. 3).

Similar results were obtained when we followed the inheritance of another complex trait, designated "clam," which appeared in *ddm1-1/ddm1-1* selfed lines. This trait is characterized by a small, compressed rosette, reduced internode length, and reduced fertility (data not shown). The inheritance of the clam phenotype in mapping crosses indicated that the trait is caused by a monogenic recessive lesion (see *Materials and Methods*). The locus responsible for the clam phenotype (*CLM*) is also unlinked from the *DDM1* locus and maps to the center of chromosome 3 (Fig. 3).

**Progressive Reduction in Cytosine Methylation in Specific Genomic Regions Parallels the Onset of Morphological Phenotypes.** The strict association between the DNA hypomethylation mutations and the onset of developmental defects prompted us to look for DNA methylation changes in selfed *ddm1/ddm1* lines. We first measured global DNA methylation levels in *ddm1/ddm1* lines during the selfing regime (see *Materials and Methods*), but the precision of these measurements was too low to reliably detect small changes in methylation levels (data not shown). We subsequently examined specific genomic regions by a more sensitive assay, Southern blot analysis, which revealed a progressive reduction in cytosine methylation during the selfing of *ddm1/ddm1* lines. Although most genomic regions known to be methylated in wild-type *A. thaliana* are hypomethylated in *ddm1* homozygotes, we previously demonstrated that two methylated single-copy regions were unaffected in *ddm1* mutants that had been selfed a limited number of generations (17). These regions are defined by two anonymous genomic clones, m105 and m118, which were identified in a survey of an *A. thaliana* genomic library for clones carrying inserts recognizing methylated *Hpa*II restriction sites (28). Fig. 4 shows that the wild-type *Hpa*II methylation pattern of the m105 locus was not affected in *ddm1/ddm1* lines that had been selfed for only one generation, confirming our previous results (17). However, stochastic loss of cytosine methylation sites at the m105 locus was noted in most *ddm1/ddm1* lines after six generations of self-pollination (Fig. 4). We identified the loss of two separate m105 methylation sites [designated epimutations 1 and 2 in Table 1; epimutations = heritable alterations in DNA modification (2)] in the selfed *ddm1/ddm1* lines (Table 1 and Fig. 4). No m105 epimutations occurred in the selfed *DDM1/DDM1* control lines. Similarly, loss of *Hpa*II site methylation (Table 1, epimutation 3) at the m118 locus occurred in many *ddm1/ddm1* lines, but not in the *DDM1/DDM1* control lines. Although the onset of particular aberrant morphological phenotypes could not be correlated with particular m105 and m118 epimutations, the stochastic loss of DNA methylation sites at these loci paralleled the progressive onset of morphological abnormalities in the selfed *ddm1/ddm1* lines.

## DISCUSSION

The results presented here indicate that loss of *A. thaliana* wild-type *DDM1* gene function leads to developmental defects. Previously we demonstrated that *ddm1* mutations, when present in a homozygous state, cause immediate hypomethylation of a large variety of repeated DNA families, as well as low-copy sequences at chromosomal termini (17). In this report, we show that the *ddm1* mutations lead to a slow loss of methylation in non-telomeric single-copy sequences. The DNA methylation system appears to operate differently on the single-copy versus repetitive genomic compartments because methylation of the two sequence classes is differentially affected by *ddm1* mutations.

A variety of morphological anomalies were generated at a high frequency in *ddm1/ddm1* lines propagated through several generations by self-pollination. The onset of the phenotypes was strictly associated with the *ddm1* mutations and never occurred in wild-type sibling lines propagated in parallel with the *ddm1* mutant lines. Although full expression of the phenotype required several generations to develop, once the morphological traits emerged, they were inherited. In each of

FIG. 2. Progressive onset of the ball phenotype in a *ddm1-2/ddm1-2* line propagated through increasing numbers of self-pollinations. (*Left*) A wild-type *DDM1/DDM1* plant, strain Columbia. All the remaining plants are Columbia *ddm1-2* homozygotes from a single line (not represented in Table 1) propagated by self-pollination. The *ddm1-2* mutant that was selfed once exhibited only mild morphological phenotypes. In the advanced selfed generations, a progressively severe ball phenotype (reduced apical dominance and plant size, twisted leaves) was evident. All plants were the same age (approximately 6 weeks) and were grown in parallel, under the same environmental conditions.

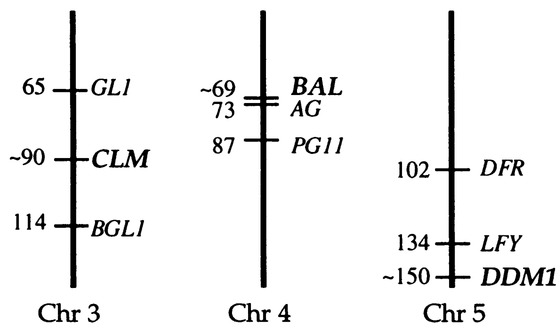


FIG. 3. Map positions of *DDM1* and the loci, *BAL* and *CLM*, affected in *ddm1* mutant backgrounds. The position of relevant reference markers are given (in centimorgans) from the recombinant inbred genetic map of Lister and Dean (27). Approximate positions for the *DDM1*, *BAL*, and *CLM* loci were determined by reference to established markers as described in the *Materials and Methods*.

the two cases examined, the complex morphological trait was caused by a heritable lesion at a single Mendelian locus unlinked to the potentiating *ddm1* mutation.

The heritable lesions underlying the phenotypes could be genetic mutations or epigenetic modifications. Three general mechanisms (not necessarily mutually exclusive) for formation of the lesions are considered here. DNA hypomethylation could lead to an increased mutation rate (model 1) due to increased transposition of previously suppressed elements (30–34) or increased recombination rates (35, 36) mediating genomic rearrangement. However, the characteristics of the phenotypic onset in the selfed *ddm1* homozygous lines cannot be easily explained by random genetic mutation events. The phenotypes occur at a high frequency in *ddm1* homozygous selfed lines, and similar phenotypes occur in independent *ddm1* mutant lines. Moreover, some phenotypes progress in severity as the number of self-pollinations increases.

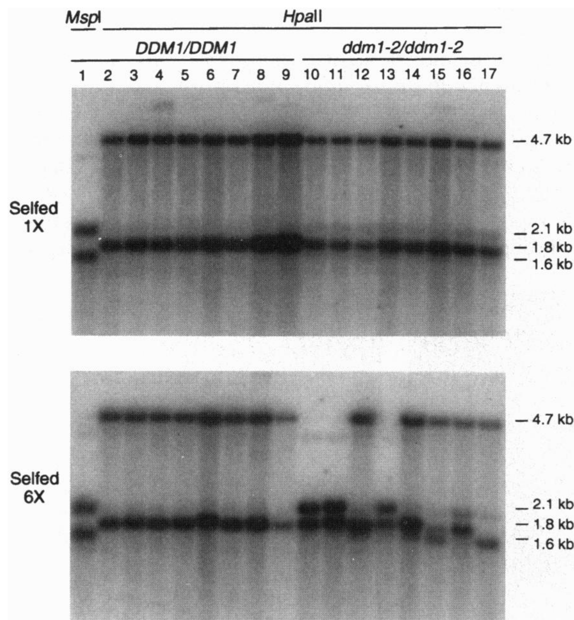


FIG. 4. Progressive loss of cytosine methylation at the m105 locus during propagation of *ddm1/ddm1* plants by self-pollination. Genomic DNA was prepared from leaf tissue from eight different *ddm1-2/ddm1-2* lines (lanes 10–17) and eight *DDM1/DDM1* control lines (lanes 1–9), which were propagated by self-pollination for one generation (*Upper*) or six generations (*Lower*). The DNA samples were digested with the methylation-sensitive restriction endonuclease *Hpa*II (lanes 2–17) or its isoschizomer, *Msp*I, which will cleave C$^{m}$CGG (29). The digested DNA samples were size-fractionated by agarose gel electrophoresis and transferred to a nylon membrane. The membrane was hybridized with a radiolabeled probe corresponding to a 3.3-kb *Eco*RI subclone from the anonymous single-copy *A. thaliana* genomic clone m105 (28). Epimutation 1 corresponds to the loss of methylation at a *Hpa*II site that converts the 4.7-kb fragment to a 2.1-kb fragment. Epimutation 2 signifies conversion of the 1.8-kb fragment to the 1.6-kb fragment by loss of *Hpa*II site methylation. Faint 2.1- and 1.6-kb bands are visible in the *ddm1-2* mutant lanes after one generation of self-pollination, possibly reflecting some loss of methylation in the vegetative tissue.

The characteristics of phenotypic onset suggest the operation of an epigenetic mechanism. In this context, we consider epigenetic modifications to be mitotically transmissible alterations that affect the expression of the locus without changing primary DNA sequence (2). One possible epigenetic mechanism (model 2) involves alteration in chromatin structure secondary to changes in cytosine methylation (37–39). Position-effect variegation in *Drosophila* provides one example of epigenetic defects based on the propagation of altered chromatin structures (40).

Another possible epigenetic mechanism for the onset of morphological phenotypes in selfed *ddm1* lines is the formation of epimutations (model 3). The slow loss of cytosine methylation in the m105 and m118 loci suggests that similar stochastic methylation site loss could create epimutations in *ddm1* backgrounds at loci distributed throughout the genome. Accumulated loss of multiple methylation sites at a single locus may be responsible for the delayed onset and progressive severity of the morphological defects. The variation in phenotypic severity seen among siblings in selfed populations could be due, in part, to continued creation of new epimutations in somatic tissue followed by transmission to and segregation in the next generation. Our group (17) and others (41, 42) have demonstrated that hypomethylated DNA is inherited in *A. thaliana* across generations due to slow *de novo* methylation. It should be noted that there is precedence for creation and transmission of stable epimutations or epigenetic states in plants (25, 43–48).

It is also possible that the *ddm1* mutations lead to the morphological phenotypes through a DNA methylation-independent pathway, but several considerations suggest that the loss of cytosine methylation is important for the delayed-onset morphological phenotypes. Phenotypes resembling the *ddm1* induced delayed-onset defects are seen in transgenic *A. thaliana* expressing cytosine methyltransferase anti-sense constructs (41, 42). In addition, dwarf-like phenotypes (reduced stature, reduced apical dominance) have been induced in *A. thaliana* (J.A.J., unpublished work) and other flowering plants using DNA methylation inhibitors (49, 50).

We are currently pursuing the molecular characterization of lesions at *ddm1* target loci, such as *BAL*, to determine the mechanism(s) responsible for the morphological defects. We anticipate that these studies will contribute to an understanding of the role of DNA methylation in eukaryotic cells.

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