The DNA methylation locus *DDM1* is required for maintenance of gene silencing in *Arabidopsis*

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To investigate the relationship between cytosine methylation and gene silencing in *Arabidopsis,* we constructed strains containing the *ddm1* hypomethylation mutation and a methylated and silenced *PAI2* tryptophan biosynthetic gene (*MePAI2*) that results in a blue fluorescent plant phenotype. The *ddm1* mutation had both an immediate and a progressive effect on *PAI* gene silencing. In the first generation, homozygous *ddm1 MePAI2* plants displayed a weakly fluorescent phenotype, in contrast to the strongly fluorescent phenotype of the *DDM1 MePAI2* parent. After two generations of inbreeding by self-pollination, the *ddm1/ddm1* lines became nonfluorescent. The progressive loss of fluorescence correlated with a progressive loss of methylation from the *PAI2* gene. These results indicate that methylation is necessary for maintenance of *PAI* gene silencing and that intermediate levels of DNA methylation are associated with intermediate gene silencing. The results also support our earlier hypothesis that *ddm1* homozygotes act as "epigenetic mutators" by accumulating heritable changes in DNA methylation that can lead to changes in gene expression.

[*Key Words:* Phosphoribosylanthranilate isomerase; *PAI;* gene silencing; epigenetics; DNA methylation; *ddm1*]

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Gene silencing phenomena are widespread among eukaryotes and have been studied extensively in higher plants (Matzke and Matzke 1993; Meyer and Saedler 1996; Depicker and Montagu 1997). Silencing of introduced transgenes is common in plants and much of the work on epigenetic regulation has focused on transgenic systems. However, gene silencing is not restricted to transgenes, as demonstrated by several examples of endogenous gene silencing in maize (Cocciolone and Cone 1993; Patterson et al. 1993; Das and Messing 1994; Hollick et al. 1995; Kermicle et al. 1995), soybean (Todd and Vodkin 1996), and *Arabidopsis* (Bender and Fink 1995; Jacobsen and Meyerowitz 1997). In some cases, transcription initiation from the silenced gene is not affected, and the loss of expression is thought to occur at the level of transcript processing or degradation (Metzlaff et al. 1997; Ratcliffe et al. 1997; Tanzer et al. 1997). In other cases, silencing occurs at the transcriptional level (Meyer et al. 1993; Patterson et al. 1993; Ye and Signer 1996). In many examples of transcriptional silencing, there is a correlation between cytosine methylation (5-MeC) of the silenced gene promoter and a loss of expression (Meyer et al. 1993; Ye and Signer 1996). Gene silencing also occurs in organisms that lack DNA methylation, such as *Drosophila* (Dorer and Henikoff 1994; Wallrath and Elgin 1995; Pal-Bhadra et al. 1997; Pirrotta 1997) and budding and fission yeasts (Pillus and Rine 1989; Aparicio et al. 1991; Allshire et al. 1994; Grewal and Klar 1996) calling into question whether DNA methylation is essential for gene silencing or whether it serves as an auxiliary reinforcement mechanism in organisms with methylated genomes.

The higher plant *Arabidopsis* provides an ideal model system for studying the role of cytosine methylation in gene expression and development of multicellular eukaryotes. Genetic tools are available in *Arabidopsis* to manipulate DNA methylation levels. *Arabidopsis* DNA hypomethylation mutants [*ddm* (Vongs et al. 1993)] and cytosine methyltransferase–antisense transgenic lines (Finnegan et al. 1996; Ronemus et al. 1996) have been developed that are viable and fertile despite displaying an array of morphological abnormalities (Finnegan et al. 1996; Kakutani et al. 1996; Ronemus et al. 1996; Richards 1997). In contrast, mouse methyltransferase-deficient mutants die during early embryogenesis (Li et al. 1992). Another advantage of *Arabidopsis* is the availability of an endogenous methylated *Arabidopsis* gene, *MePAI2,* whose silenced, fluorescent phenotype can be easily monitored by visual inspection throughout the development of the plant (Bender and Fink 1995). Furthermore, the intensity of the fluorescent phenotype, which reflects the level of *MePAI2* silencing, can be quantitated.

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PAI2 is one of four *PAI* sister genes in the Wassilewskija (WS) strain of *Arabidopsis* that encodes the third enzyme in the tryptophan biosynthetic pathway, phosphoribosylanthranilate isomerase (*PAI*). In WS, the four *PAI* genes are located at three unlinked sites in the genome (Fig. 1) (Bender and Fink 1995). All four genes are heavily cytosine-methylated over their regions of shared DNA sequence similarity. The combined expression of the four methylated *PAI* (*MePAI*) genes in WS provides enough PAI activity for a normal plant phenotype. However, in a mutant where two tandemly arrayed *PAI* genes, *MePAI1–MePAI4*, are deleted, the two remaining genes, *MePAI2* and *MePAI3*, provide insufficient *PAI* activity for normal development. A striking PAI-deficient phenotype displayed by the *Δpai1–pai4* deletion mutant is blue fluorescence under UV light, caused by accumulation of early intermediates in the tryptophan pathway, anthranilate and anthranilate-derived compounds (Last and Fink 1988; Bender and Fink 1995; Li et al. 1995).

Several lines of evidence suggest that the residual methylation on the *PAI2* gene in the fluorescent *pai* mutant is associated with PAI-deficient phenotypes. First, the fluorescent *pai* mutant gives rise to spontaneous nonfluorescent revertant progeny at 1%–5% per generation, and in these revertant lines there is substantial hypomethylation of both *PAI2* and *PAI3* (Bender and Fink 1995). Spontaneous partial revertant lines with intermediate levels of fluorescence have also been isolated, and these lines display partial hypomethylation (J. Bender, unpubl.; see Results). Furthermore, growth of the fluorescent *pai* mutant on the cytosine methyltransferase-inhibiting compound 5-azacytidine relieves the silenced fluorescent phenotype (Bender and Fink 1995). Because the *MePAI3* locus is not linked to the fluorescent phenotype when segregated through genetic crosses (Bender and Fink 1995), and because the *PAI3* gene has very low expression levels even when unmethylated (Li et al. 1995), the *MePAI2* locus is the critical determinant for the blue fluorescent PAI-deficient phenotype. Therefore, *MePAI2* serves as a facile reporter for methylation-correlated gene silencing in *Arabidopsis*.

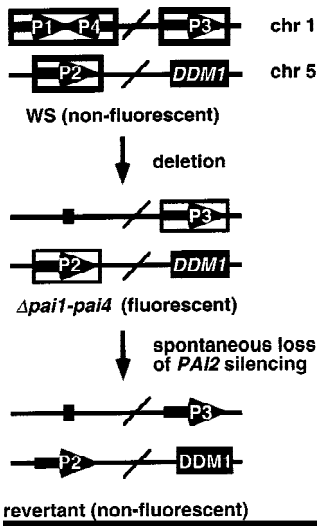

Figure 1. *PAI* gene organization. The organization of the four *PAI* genes in *Arabidopsis* strain WS is shown. The arrows depict the direction of transcription. The thickness of the lines surrounding each gene reflects the density of cytosine methylation at each locus. The slash indicates that the genes are on the same chromosome but are genetically unlinked.

In this report we combine the *Arabidopsis ddm1* DNA hypomethylation mutation with the *MePAI2*-silenced reporter gene to carry out a genetic analysis of methylation and silencing. *ddm1* mutations cause an immediate loss of modification in repeated DNA when first made homozygous and foster a progressive loss of methylation in the low-copy portion of the genome over several generations of inbreeding (Vongs et al. 1993; Kakutani et al. 1996). Use of the hypomethylation mutation allows more precise control over DNA methylation than is possible with methylation inhibitors and provides an opportunity to examine gene silencing within the developmental context of whole plants.

Results

ddm1 *suppresses the silenced fluorescent phenotype of the* pai *mutant*

To assess the effect of the DNA hypomethylation mutation *ddm1* on *PAI2* gene silencing, we introduced *ddm1* into the fluorescent *pai* mutant background as shown in Fig. 2. We identified several blue fluorescent F_2 individuals from a cross between the fluorescent *pai* mutant (*Δpai1–pai4/Δpai1–pai4; MePAI2/MePAI2* in the WS background) and a homozygous *ddm1* mutant strain (*ddm1-2/ddm1-2* in the Columbia strain). The F_2 fluorescent segregants were homozygous for the recessive *Δpai1–pai4* deletion and the recessive, methylated, and silenced *MePAI2* locus from the *pai* mutant parent. We then screened the fluorescent F_2 segregants with a polymorphic marker, m555, which is tightly linked to the *ddm1* mutation (within 1 cM; J.A. Jeddeloh, unpubl.) to determine the *ddm1* genotype of each line. One representative fluorescent segregant that was heterozygous for the m555 marker (and thus heterozygous *DDM1/ddm1-2*) was used for subsequent detailed analysis.

The representative fluorescent *DDM1/ddm1-2* heterozygous F_2 isolate, designated *pai d/D1*, was allowed to self-pollinate. The segregation patterns of the fluorescent silenced phenotype in the resulting F_3 population were scored relative to the m555 genotype or the genomic hypomethylation phenotype diagnostic of *ddm1* (Vongs et al. 1993). Three phenotypes were seen in F_3 populations segregating *ddm1:* strongly fluorescent (the parental *pai* mutant phenotype), weakly fluorescent (a nonparental phenotype), and nonfluorescent (a spontaneous revertant phenotype) (Fig. 3; Table 1). F_3 progeny from three other *DDM1/ddm1-2* heterozygous fluorescent F_2 segregants showed similar patterns of phenotypes (data not shown).

The strongly fluorescent phenotype (64/90 plants scored = 71%) corresponded to F_3 plants that carried

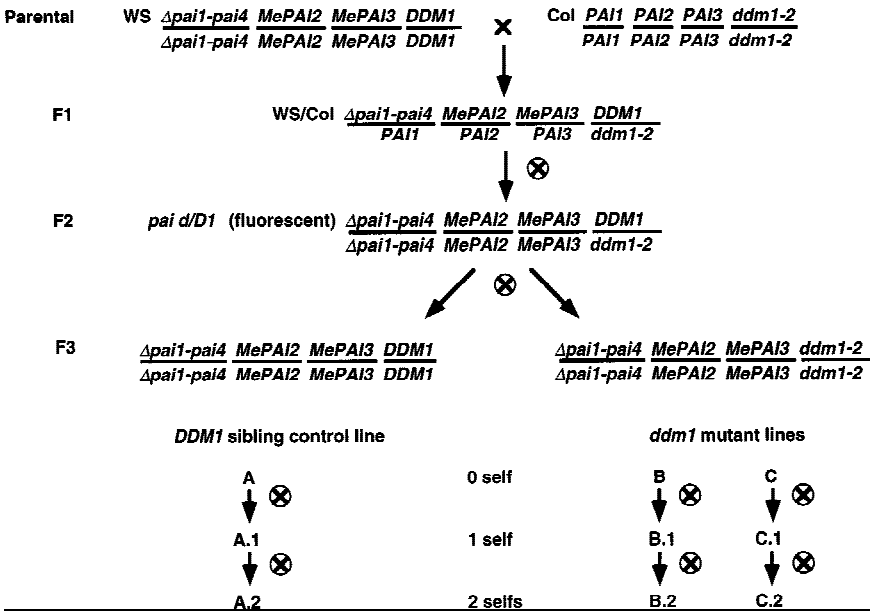

Figure 2. Genetic pedigrees used to construct *Δpai1–pai4 ddm1* double mutants and control lines.

the wild-type *DDM1* WS allele (*DDM1/DDM1* and *DDM1/ddm1-2*) (Table 1). All plants that displayed the nonparental weakly fluorescent phenotype (23/90 plants scored = 26%) were homozygous for the *ddm1-2* Columbia allele. One of three nonfluorescent plants (1/90 = 1%) was also homozygous for the *ddm1-2* allele. The remaining two nonfluorescent plants (2/90 = 2%) carried the WS *DDM1* allele and represent spontaneous nonfluorescent revertants of the *MePAI2* silent state, which were previously determined to segregate from the fluorescent *pai* mutant at 1%–5% per generation (Bender and Fink 1995). Therefore, plants homozygous for the recessive *ddm1* mutation display an immediate suppression of the fluorescent silenced *pai* phenotype.

Intermediate silencing in pai ddm1 *double mutants*

Examination of the developmental context of the fluorescence phenotype provided insight into the effects of *ddm1* on *PAI2* gene silencing. In our segregating F_3 populations, *pai DDM1* mutant individuals were either fluorescent throughout the plant or displayed occasional nonfluorescent unsilenced sectors on leaves and stems (Bender and Fink 1995). We have not observed small patches of fluorescent cells in fields of nonfluorescent cells. These results suggest that loss of silencing events during development are common, whereas shifts from a nonsilenced to a silenced state are extremely rare or do not occur.

The *pai ddm1* double mutant lines displayed similar sectoring patterns except that their fluorescent tissue was less bright (Fig. 3). The weakly fluorescent phenotype could result from intermediate levels of *PAI2* gene silencing giving rise to intermediate levels of anthranilate compounds within each cell in the sector. Alternatively, *PAI2* gene silencing might be constrained to one of two states, fully silenced or nonsilenced. In this model, mixtures of nonfluorescent (unsilenced) and fully

Figure 3. Fluorescence phenotypes of *Δpai1–pai4* mutants in different genetic backgrounds. (*Left*) Genotypes of the plants photographed under short-wave UV (*center*) and white light (*right*), respectively.

Table 1. Δpai1–pai4 ddm1 *double mutants express a nonparental weakly fluorescent phenotype*

Phenotype	Genotype		Totals
	DDM1/_	*ddm1/ddm1*	
Strongly fluorescent	64	0	64
Weakly fluorescent	0	23	23
Nonfluorescent	2 (revertant class)	1	3
Totals	66	24	90

ddm1 mutants and nonmutants (*DDM1/_*) were identified in a *Δpai1–pai4 MePAI2 MePAI3* background (F_3 generation, Fig. 2) after determination of their fluorescence phenotype. The segregation data include two independent plantings of the same family. An association between the *ddm1* genotype and the fluorescence phenotype is indicated by a χ^2 statistic of ~85, $P < 0.001$, $df = 2$, taking a lack of association between the *ddm1* genotype and the fluorescence phenotype as a null hypothesis.

fluorescent (silenced) cells would give the appearance of weak fluorescence at a distance.

Two lines of evidence support the intermediate silencing model. First, the relatively large weakly fluorescent sectors seen in *pai ddm1* double mutants resembled those from a spontaneously derived partial revertant *Δpai1–pai4* line (*REVpart*) (Fig. 3). The large sector sizes reflect relatively infrequent shifts from the silenced to nonsilenced state early in leaf development. The two-state model must invoke an additional hypersectoring phase later in development to generate the predicted mixture of strongly fluorescent and nonfluorescent cells. Second, the weakly fluorescent sectors in *pai ddm1* double mutants and *REVpart* were homogeneous. No microsectors of nonfluorescent and strong fluorescent cells were visible within the weakly fluorescent sectors. Homogeneity for the fluorescence phenotype was also demonstrated at the cellular level. FACS analysis indicated that weakly fluorescent *pai ddm1* and *REVpart* plants consist only of populations of intermediate- and nonfluorescent cells, with no indication of a subpopulation of strongly fluorescent cells predicted by the two-state model (Fig. 5B, below). A bimodal distribution of strongly and nonfluorescent cells were seen in cell populations derived from strongly fluorescent *pai DDM1* control plants (Fig. 5B, below). Such a bimodal distribution suggests that the anthranilate compounds do not readily diffuse between cells inside the plant, consistent with our previous observation that the sectors have sharp boundaries (Bender and Fink 1995). Therefore, it is likely that the fluorescent phenotype is cell autonomous. These considerations suggest that the majority of cells in the fluorescent tissues of newly segregated *pai ddm1* double mutant lines have an intermediate level of silencing that results in an intermediate fluorescent phenotype.

pai ddm1 *double mutants have reduced accumulation of fluorescent anthranilate compounds*

PAI2 gene silencing in *ddm1* mutant and *DDM1/_* F_3 individuals was quantitated by measuring the accumulated PAI substrates, anthranilate compounds, using fluorometric detection (Fig. 4). F_3 *pai ddm1* homozygotes had levels of anthranilate compounds about sixfold less than the *pai DDM1* siblings, consistent with the qualitative scoring shown in Table 1. No significant differences were seen between *DDM1/ddm1-2* and *DDM1/DDM1* plants in the amount of fluorescence (data not shown). The large standard deviations seen in the fluorescence measurements are expected from sampling tissues with large fluorescent/nonfluorescent sectors.

The level of fluorescence in F_3 *ddm1* mutants was significantly higher than either a spontaneous nonfluorescent revertant line, *REV2* (Bender and Fink 1995), or the Columbia *ddm1* mutant donor. This finding suggests that the *ddm1* mutants contain residual *PAI2* silencing, whereas spontaneous nonfluorescent revertants exhibit essentially no *PAI2* silencing.

Inbreeding ddm1 *mutants progressively extinguishes* PAI2 *silencing*

Because *ddm1* mutations cause inbreeding-associated progressive DNA hypomethylation, we investigated the effect of inbreeding *pai ddm1* mutants. From the segregating F_3 family we started two *pai ddm1* mutant lines B and C, as well as a sibling *pai DDM1* control line A (Fig. 2). As shown in Figure 5A, inbreeding *pai ddm1* mutants

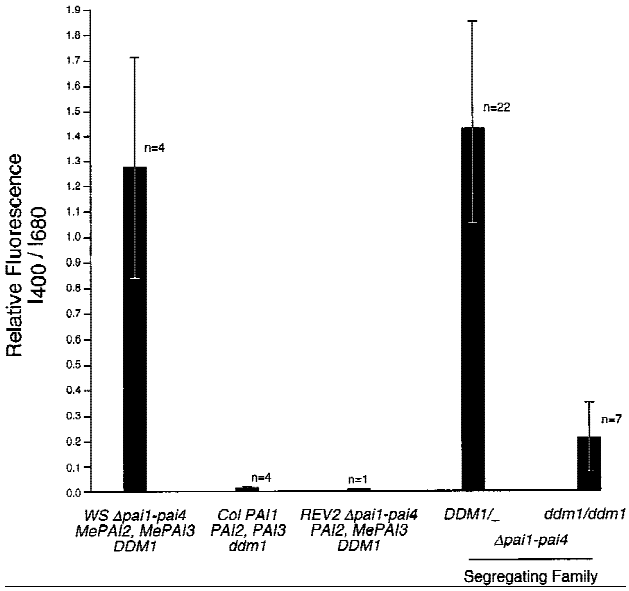

Figure 4. Quantifying *PAI2* gene silencing by measurement of anthranilate compounds. Accumulated anthranilate and anthranilate compounds in leaves of plants with the indicated genotypes were measured spectrofluorometrically. Values were normalized to chlorophyll fluorescence. The histogram displays the mean value for the particular genotype and the standard deviation. (*n*) The number of independent leaf extractions and quantitations. Five leaves from individual plants were extracted for the control genotypes; two leaves were extracted per individual in the *ddm1* segregating family.

led to a progressive loss of residual *PAI2* gene silencing. The loss of fluorescence followed different trajectories in *ddm1* lines B and C, suggesting that the loss of silencing is stochastic. The inbreeding effects are specific to *ddm1* mutants because no significant changes in fluorescence levels were seen upon inbreeding the *pai DDM1* control line A.

ddm1 *induces progressive hypomethylation of silenced* PAI *genes*

To investigate whether the *ddm1-2* mutation affects *PAI2* gene silencing through a reduction in DNA methylation, we used cytosine methylation-sensitive restriction enzymes and Southern blot analysis to determine the DNA methylation status of the *PAI2* and *PAI3* genes in representative *pai* mutant lines (Figs. 2 and 5). As shown in Figure 6, the *PAI* genes in the fluorescent *pai DDM1* control DNA samples showed moderate to heavy methylation of all sites investigated. DNA from the spontaneous nonfluorescent revertant line, *REV2*, had hypomethylated restriction sites in *PAI2* and slight residual methylation of sites in *PAI3*. In contrast, the *ddm1* mutation caused a complex pattern of DNA hypomethylation for *PAI2* and *PAI3*. For example, Figure 6B shows that the *Hpa*II–*Msp*I (CCGG) site within the transcribed region of the *PAI3* gene was progressively hypomethylated in the *ddm1* mutant line B.1 → B.2 [$^{m}C^{m}CGG \rightarrow CCGG$; both *Hpa*II and *Msp*I (McClelland et al. 1994) are blocked in B.1]. However, there was a loss of ^{m}CpG methylation at the *Hpa*II–*Msp*I site in *PAI2* during inbreeding of *ddm1* line C.1 → C.2 without an effect on *PAI3* methylation. Methylation of *Sau*3AI–*Dpn*II sites (GAT^{m}C) within the transcribed regions of *PAI2* and *PAI3* was also reduced in the *ddm1* mutant lines but the hypomethylation was incomplete (data not shown), indicating further that the changes in methylation of different sites are independent.

The hybridization pattern shown in Figure 6C indicates that the *Pst*I sites in the transcribed regions of the *PAI2* and *PAI3* genes were progressively hypomethylated during the inbreeding of *ddm1* mutants (Fig. 6C). The 700- and 200-bp *Pst*I fragments derived from hypomethylated *PAI2* loci accumulated during the inbreeding of *ddm1* lines B.1 → B.2 and C.1 → C.2. Again, although there was a trend toward hypomethylation, the changes in *Pst*I site methylation through the inbreeding regime did not completely match the changes in *Hpa*II–*Msp*I sites or *Sau*3AI–*Dpn*II sites. The changes in *PAI2 Pst*I site modification matched the expression data shown in Figure 5 most closely.

Sequence analysis of PAI2 *hypomethylation induced by* ddm1

To obtain a more detailed picture of the *ddm1*-induced loss of methylation from *PAI2*, we employed the 5-MeC DNA sequencing protocol developed by Frommer and colleagues (1992) to examine the upstream region of *PAI2* (Figs. 7 and 8). The 5-MeC sequencing technique relies on the bisulfite-mediated conversion of cytosine, but not 5-MeC, to uracil. After bisulfite pretreatment of genomic DNA from lines A.1, C.1, and C.2, an ~400-bp region corresponding to one strand near the *PAI2* transcription start was amplified by PCR. The resulting products were cloned, and the nucleotide sequence was determined for 8–10 alleles from each line. Cytosines

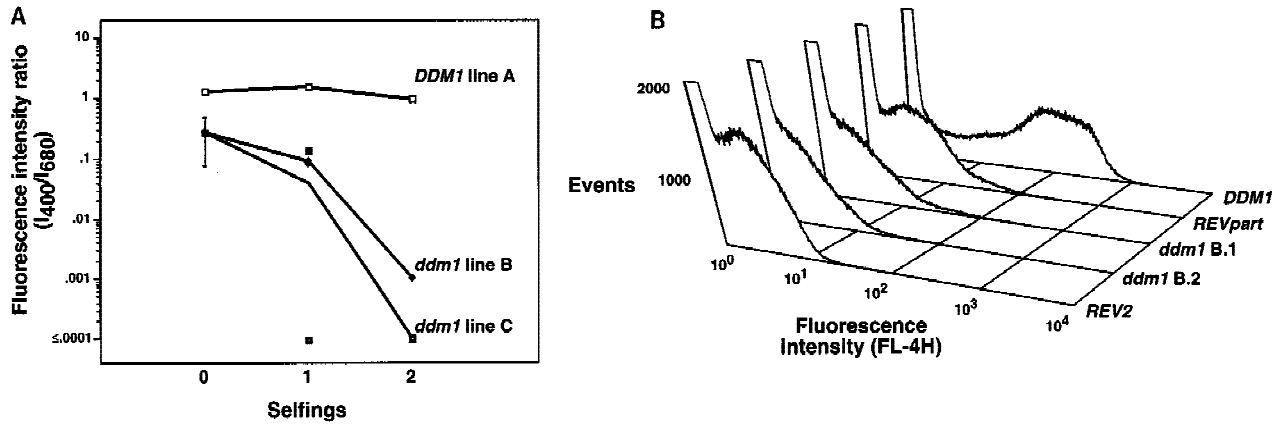

Figure 5. *ddm1* progressively extinguishes Me-PAI2 silencing. (*A*) Accumulated anthranilate and anthranilate compounds were measured spectrofluorometrically from leaves of two independent *Δpai1–pai4 ddm1* lines (B.1 → B.2 and C.1 → C.2) and a *Δpai1–pai4 DDM1* control line (A.1 → A.2) that had been selfed 0–2 generations. Values were normalized to chlorophyll fluorescence as in Fig. 4. Ten leaves from individual plants were extracted in two groups of five leaves for the one and two selfing generation data. Values for the zero selfing generation correspond to the average fluorescence measurement of *Δpai1–pai4 ddm1* or *DDM1*/_ individuals in the segregating family shown in Fig. 4. For the C.1 line, the fluorescence measurements for the two independent five leaf samples were widely disparate and both data points are shown. Such a wide variation presumably reflects a jackpot phenomenon associated with sampling tissues with large sectors. (*B*) FACS analysis of different *Δpai1–pai4* lines (see Materials and methods). Genotypes are indicated at *right*. The *y*-axis indicates number of cells/particles counted.

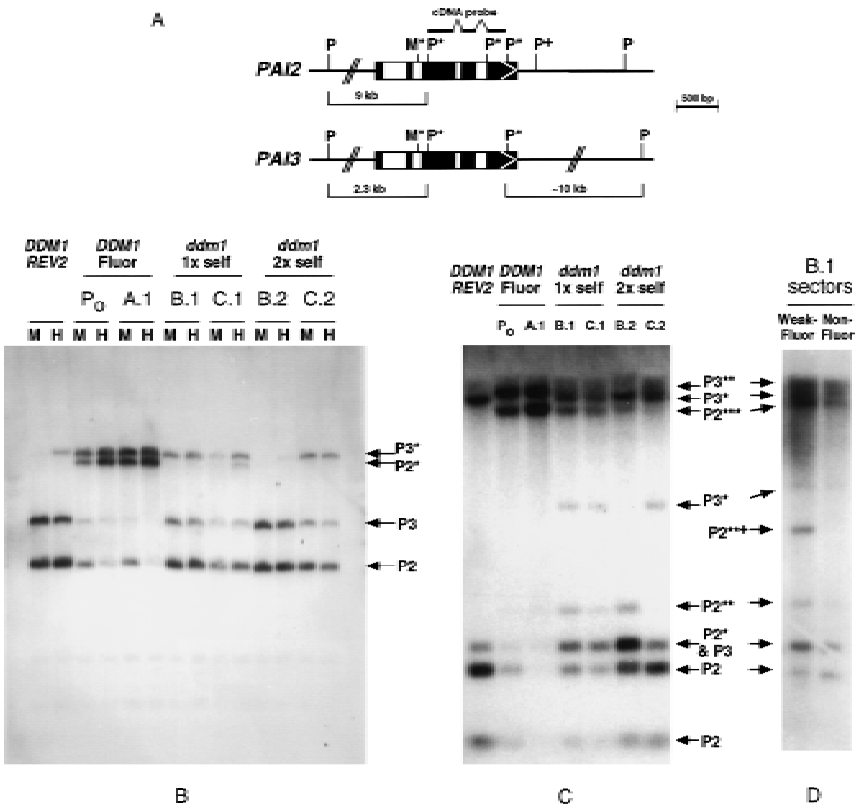

Figure 6. *ddm1* leads to progressive hypomethylation of *PAI2*. (*A*) A restriction map of the WS *PAI2* and *PAI3* loci. (M) *Msp*I–*Hpa*II; (P) *Pst*I; (* or +) methylated in the *Δpai1–pai4* parent (see Materials and Methods). Direction of transcription is indicated by the arrow. Shaded boxes correspond to exons; open boxes correspond to introns. The origin of the hybridization probe used for *B–D*, a 668-bp *Pst*I fragment from the Col cDNA corresponding to *PAI1* (containing 1 single-base difference from *PAI2* and 30-single base differences from *PAI3*), is indicated at the *top*. (*B–D*) Genomic Southern blot analysis of *ddm1* effects on *Msp*I (M), *Hpa*II (H), and *Pst*I sites (*C, D*) in *PAI2* and *PAI3*. Genotypes of the *Δpai1–pai4* lines are indicated, and the letter designations refer to the specific generations shown in Fig. 2. The origin of the fragments is shown at *right:* (P2) *PAI2;* (P3) *PAI3*. The methylation state of each allele is indicated by the superscripts [all sites marked by an asterisk (*) except for the *Pst*I site directly downstream of the *PAI2* coding region which is marked by +], with the number of symbols reflecting the number of methylated sites. (*D*) Southern analysis of genomic DNA prepared from weakly fluorescent (Weak-Fluor) and nonfluorescent (NonFluor) sectors cut from leaves of five *pai ddm1* individuals from line B.1. A novel methylated *PAI2* allele (P^{**+}) was seen in the plants used for *D*, which were planted independently from those used for *C*.

detected in the sequenced alleles correspond to unconverted 5-MeCs in the original genomic DNA.

This detailed genomic sequence analysis revealed that in weakly fluorescent *pai ddm1* double mutants there is a mixture of differentially methylated DNA alleles, whereas in nonfluorescent inbred progeny of the *pai ddm1* double mutant there is very little residual *PAI* gene methylation. In the fluorescent *pai DDM1* mutant, cytosine methylation occurs at symmetrical CpG and CpNpG sites and at asymmetrically disposed cytosines in the *PAI2* upstream region (Fig. 8). Methylation at both symmetric and asymmetric sites has been observed previously in a number of other plant sequences (Martienssen and Baron 1994; Meyer et al. 1994; Ronchi et al. 1995; Jacobsen and Meyerowitz 1997). The most heavily methylated allele from the fluorescent *pai DDM1* mutant had approximately half of the 5-MeCs at asymmetric sites, whereas less methylated alleles contained predominantly symmetrical site modification (Fig. 8). In all of the sequenced alleles, methylation was heaviest from ~80-bp upstream of the transcription start site extending into the transcribed region of the *PAI2* gene. Also, in none of the sequenced alleles was methylation found >210 bp upstream of the transcription start site, consistent with previous determinations from Southern blot analysis that *PAI* methylation in the *pai* mutant and in parental WS does not spread significantly beyond the boundaries of shared sequence similarity among sister *PAI* genes (Bender and Fink 1995). Four of five sequenced alleles from the spontaneous nonfluorescent revertant strain *REV2* had essentially no methylation, whereas the fifth allele is hypermethylated (Figs. 7 and 8). Again, this sequencing analysis is consistent with previous Southern blot analysis of methylation patterns in *REV2*, which indicate slight residual methylation of the *PAI2* gene can occur in this line (Bender and Fink 1995).

The *ddm1* mutation caused a reduction in methylated sites throughout the *PAI2* upstream region relative to the *pai DDM1* fluorescent strain (Fig. 7; cf. A.1 and C.1). In DNA prepared from weakly fluorescent *pai ddm1* double mutant plants (line C.1), 7 of 10 *PAI2* alleles sequenced had no or very low levels of methylation, 2 of 10 alleles had moderate methylation, and 1 of 10 alleles remained heavily methylated (Fig. 8). In the low and moderately methylated alleles, only 2 of 25 methylated sites were in asymmetric positions, whereas in the one heavily methylated allele 15 of 33 methylated sites were in asymmetric positions. Inbreeding the *pai ddm1* mutants led to an almost complete loss of DNA methylation in the *PAI2* upstream region (cf. C.1 and C.2). The

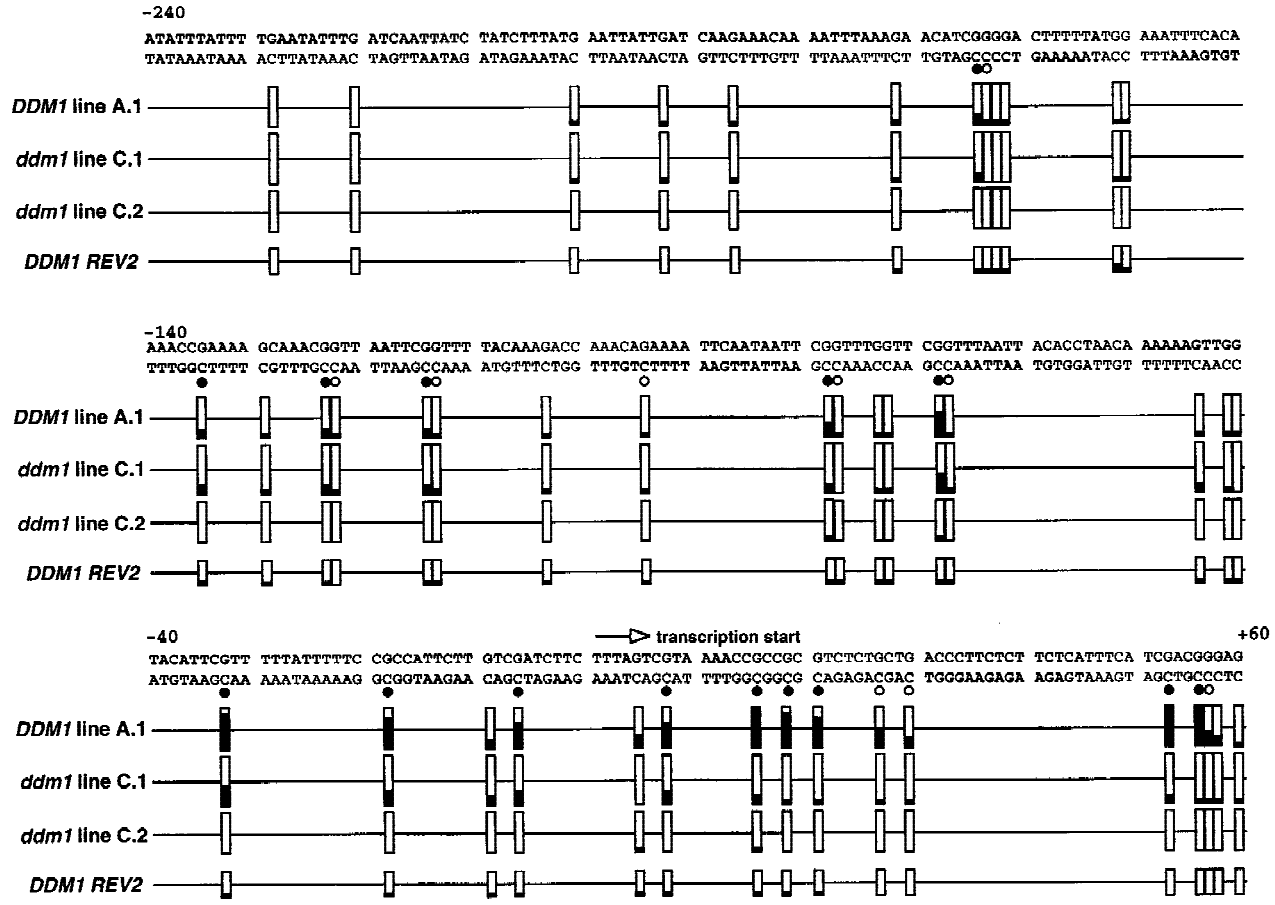

Figure 7. High-resolution methylation mapping in the *PAI2* upstream region shows that *ddm1* leads to progressive hypomethylation. The methylation status of cytosines in the *PAI2* upstream region was determined by bisulfite DNA sequencing. Approximately 300 bp of double-stranded sequence is shown corresponding to the area surrounding the transcription start site (arrow) of the *PAI2* gene. Genotypes of the *Δpai1–pai4* lines are indicated (see Fig. 2). Each cytosine on the bottom strand sequence is indicated by a rectangle whose size reflects the number of individual clones sequenced: (A.1) 8 clones; (C.1) 10 clones; (C.2) 8 clones; (*REV2*) 5 clones. The degree to which each rectangle is filled reflects the methylation occupancy at that site. (●) Cytosines in symmetrical sites; (○) cytosines in nonsymmetrical sites.

pattern of progressive hypomethylation of the *PAI2* promoter in *ddm1* line C.1 → C.2 (Figs. 7 and 8) and the expression data shown in Figure 5A suggest that the loss of *PAI2* gene silencing in the C.0 → C.1 → C.2 line is connected to the methylation loss.

It seemed likely that the mixture of differentially methylated alleles in the weakly fluorescent *pai ddm1* C.1 double mutant reflects the fluorescence sectoring phenotype, with the more methylated alleles corresponding to the weakly fluorescent sectors and the sparsely methylated alleles corresponding to nonfluorescent sectors. To test this hypothesis, we dissected weakly fluorescent and nonfluorescent sectors from weakly fluorescent *pai ddm1* double mutants and extracted DNA for Southern blot analysis of methylation patterns. This analysis revealed that the *PAI* genes from fluorescent sectors had higher methylation than *PAI* genes prepared from nonfluorescent sectors (Fig. 6D), consistent with a correlation between DNA methylation and gene silencing even within the tissues of the same plant.

Discussion

Cytosine methylation is necessary for PAI2 *gene silencing*

Our findings address the relationship between DNA methylation and gene silencing, as well as the mode of action of *Arabidopsis ddm1* DNA hypomethylation mutations. The *ddm1-2* mutation was used to progressively reduce the methylation levels of the silenced *PAI2* gene. We found that the progressive loss of methylation correlates with a progressive loss of gene silencing. Hypermethylated alleles recovered from *pai ddm1* mutant line C.1 and the nonfluorescent revertant control line *REV2* do not violate the strict correlation between cytosine methylation and gene silencing because infrequent silenced, methylated alleles will be recessive to expressed alleles (Bender and Fink 1995; J. Bender, unpubl.). In no case did we find silencing to persist in the absence of methylation.

There are two possible general models to explain the effect of *ddm1* mutations on gene silencing. The sim-

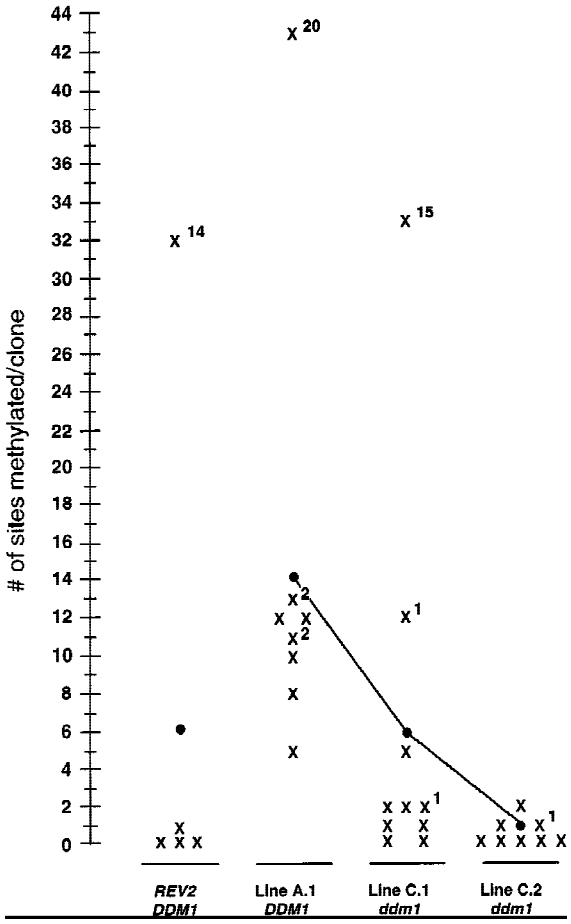

Figure 8. Distribution of cytosine methylation in independent *PAI2* clones derived from different *Δpai1–pai4* backgrounds. The superscripts on the X's indicate the number of methylated cytosines at nonsymmetrical sites within the clone. (●) The mean number of methylated cytosines per genotype.

plest model is that *ddm1* mutations suppress gene silencing directly through a reduction in DNA methylation of the silenced loci. The alternative model is that *ddm1* mutations affect a central process, such as chromatin structure, which leads to two independent consequences: DNA hypomethylation and the loss of gene silencing. The progressive coordinate reduction in gene silencing and DNA methylation demonstrated here in self-pollinated (or inbred) *pai ddm1* lines is most consistent with the first model. Further evidence for a direct connection between silencing and methylation comes from the recent isolation of several *Arabidopsis* mutations that suppress transgene silencing which leads to a general genomic hypomethylation (including new *ddm1* alleles) (Mittlesten Scheid et al. 1998). In addition, reduction of *PAI2* DNA methylation using the methylation inhibitor 5-azacytidine, rather than *ddm1* mutations, also leads to a loss of *PAI2* gene silencing (Bender and Fink 1995). All available data indicate that DNA methylation is necessary, if not sufficient, for *PAI2* silencing and suggest that DNA modification participates as an integral part of the silencing process.

The correspondence between the intermediate levels of *PAI2* DNA methylation and intermediate silencing suggests that cytosine methylation can cement a transcriptional state in a position between fully expressed and fully silenced. There are precedents for the establishment and propagation of intermediate epigenetic states in a number of systems, including *Saccharomyces cerevisiae* (Sherman and Pillus 1997), *Schizosaccharomyces pombe* (Allshire et al. 1994), *Ascobolus immersus* (Colot and Rossingnol 1995), *Neurospora crassa* (Irelan and Selker 1997), *Drosophila* (Wallrath and Elgin 1995), *Antirrhinum majus* (Bollmann et al. 1991), maize (Patterson et al. 1993; Hollick et al. 1995; Kermicle et al. 1995), and *Arabidopsis* (Davies et al. 1997). In some cases, intermediate epigenetic states have been tied to intermediate methylation levels (Colot and Rossingnol 1995; Davies et al. 1997; Irelan and Selker 1997; E. Walker, pers. comm.). An attractive mechanistic hypothesis for the role of methylation in silencing is that 5-MeC modification provides a mark on particular genomic regions that promotes the assembly of other factors that block transcription (Kass et al. 1997). By this model, intermediate levels of methylation could promote intermediate densities of silencing factors leading to intermediate effects on transcription.

Maintenance of PAI2 *methylation*

The methylation analysis of *PAI2* reported here addresses the mechanism by which DNA methylation patterns are propagated. Two types of cytosine methyltransferase activities have been differentiated: a de novo activity that can methylate unmethylated substrate DNA, and a maintenance activity that can methylate hemimethylated substrate DNA such as the species that are generated after replication of regions that were previously methylated de novo (Holliday and Pugh 1975; Riggs 1975). Theoretical considerations suggest that maintenance methylation is specific for symmetrical sites (CG and CNG) and data from transformation experiments in plants (Weber et al. 1990) and mammals (Wigler et al. 1981) support these considerations. The methylated *PAI2* and *PAI3* genes in the fluorescent *Δpai1–pai4* deletion mutant are likely to be relics of a de novo methylation event in the parental strain WS that persist solely through efficient maintenance methyltransferase activity (Fig. 1) (Bender and Fink 1995). This conclusion is supported by our observations that the transition from the silenced to the nonsilenced state appears to be unidirectional in vegetative tissues. Furthermore, spontaneously hypomethylated nonfluorescent revertant lines generated from the fluorescent *Δpai1–pai4* mutant do not segregate progeny that have returned to the methylated and silenced fluorescent state de novo at a detectable frequency even after several generations (Bender and Fink 1995; J. Bender, unpubl.). The recovery of a hypermethylated *MePAI2* allele from *REV2* genomic DNA, however, suggests that de novo methylation may occur at a low frequency. Such events might be restricted to particular cell types (e.g., polyploid cells) or cell lin-

eages ineligible to be incorporated into the reproductive tissues.

The maintenance methylation of the *PAI2* promoter region in the fluorescent *pai* mutant occurs mainly at symmetrically disposed cytosines with occasional asymmetric methylation sites (Fig. 7). These patterns suggest that maintenance methylation of symmetrical sites might occasionally potentiate methylation of asymmetric sites by a de novo activity. Alternatively, the maintenance methyltransferase activity in *Arabidopsis* might be capable of recognizing both symmetric and asymmetric cytosines. This maintenance activity might be relatively nonspecific in its selection of substrate cytosines, using the presence of 5-MeC residues on the old strand of DNA as a signal to methylate cytosines in the general area on the newly synthesized opposite strand of DNA after each round of replication.

The ddm1 *mutation compromises maintenance methylation*

We propose that *ddm1*-induced loss of *PAI2* silencing is mechanistically related to the loss of silencing observed in spontaneous nonfluorescent revertants of the fluorescent *pai* mutant (Fig. 9). The fluorescent *pai* mutant gives rise to spontaneous nonfluorescent or weakly fluorescent revertant progeny at 1%–5% per generation (Bender and Fink 1995). In contrast, *pai ddm1* double mutants are all weakly or nonfluorescent, and within one to two generations of inbreeding, the double mutants become nonfluorescent. The rate of spontaneous decay of the silenced state is increased by loss of *DDM1* function. The simplest explanation is that in both cases, the loss of silencing results from a breakdown in maintenance methylation that results in hypomethylated *PAI2* alleles.

The progressive loss of methylation from *PAI2* and *PAI3* in the *pai ddm1* double mutant suggests that the *ddm1* mutation compromises the fidelity or efficiency of the maintenance methylation system. Our previous results indicated that *ddm1* mutations do not affect extractable DNA methyltransferase activity or the metabolism of the activated methyl group donor, S-adenosylmethionine (Kakutani et al. 1995). The function of the wild-type *DDM1* gene product could be to recruit the cytosine methyltransferase to the replication foci, or the *DDM1* product could be a structural protein that acts at the interface between chromatin and the methylation machinery.

The ddm1 *mutation acts as an epigenetic mutator*

We previously showed that *ddm1* mutants display a spectrum of dramatic phenotypic abnormalities after inbreeding homozygous lines for several generations (Kakutani et al. 1996). In some cases, morphological phenotypes become progressively more severe over several inbred generations. Genetic mapping experiments demonstrate that the phenotypes that emerge in *ddm1* inbred lines are the result of lesions at loci unlinked to the

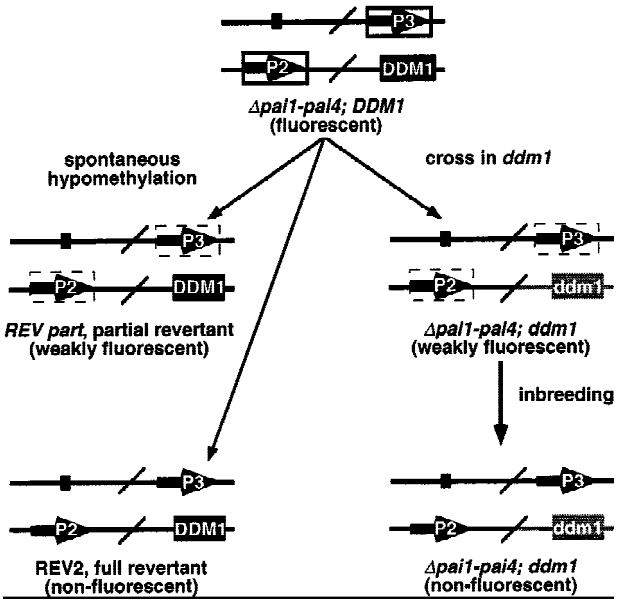

Figure 9. Model for the interaction between DNA methylation and *PAI* gene silencing. The *PAI2* and *PAI3* genes in a *Δpai1–pai4* mutant background are depicted as in Fig. 1. Dashed boxes around genes indicate intermediate levels of methylation. Loss of *MePAI2* silencing can occur by two pathways: (1) a low frequency spontaneous loss of silencing to generate partially fluorescent or nonfluorescent revertant progeny; and (2) *ddm1*-induced progressive loss of silencing at high frequency.

potentiating *ddm1* mutation. These lesions are stable in the absence of *ddm1*. The high frequency of occurrence, progressive severity, and limited spectrum of defects observed in inbred *ddm1* lines are most consistent with the hypothesis that the *ddm1*-induced lesions are epigenetic in origin and do not reflect traditional genetic mutations. These considerations led to the proposal that *ddm1* lines acts as ''epigenetic mutators'' by causing cumulative loss of 5-MeC from sensitive loci that could lead to alterations in gene expression (Kakutani et al. 1996; Richards 1997). Because *Arabidopsis* has a slow rate of de novo methylation (Vongs et al. 1993; Kakutani et al. 1995; Finnegan et al. 1996; Ronemus et al. 1996), progressively hypomethylated loci created in *ddm1* backgrounds can segregate during inbreeding. Consistent with the epigenetic mutator model, *ddm1* promotes a progressive reduction in cytosine methylation of *PAI2* and *PAI3* and a corresponding progressive increase in *PAI2* expression during inbreeding of *pai ddm1* mutants.

The behavior of *PAI* loci in *ddm1* backgrounds suggests that other silenced genomic loci would be susceptible to ectopic expression in *ddm1* inbred lines due to altered methylation of sites near or within the silenced gene. A breakdown in gene silencing could lead to developmental defects directly through gene misexpression. DNA hypomethylation could also mediate changes in expression of more distant loci by alteration of chromatin domains, chromatin boundaries, or three-dimensional interactions (Dernburg et al. 1996). DNA hypo-

methylation of transposable elements dispersed throughout the genome could also lead to inappropriate expression of neighboring genes (Martienssen and Richards 1995; Martienssen 1996; Yoder et al. 1997), as has been observed in several cases in maize (Banks et al. 1988; Martienssen et al. 1990; Martienssen and Baron 1994) and the mouse (Michaud et al. 1994). Another possibility is that genomic hypomethylation may trigger local hypermethylation of certain loci leading to developmental defects, as has been shown recently by methylation analysis of a floral homeotic gene segregating from a methyltransferase antisense transgenic line (Jacobsen and Meyerowitz 1997). Regardless of the specific mechanism(s), further study of *ddm1*-induced defects and the *DDM1* gene will lead to a better understanding of how DNA methylation is involved in maintenance of epigenetic genomic information.

Materials and methods

Plant growth

Plants were grown in a mixture of Redi-Earth (Scotts)/vermiculite (60%:40%) in environmental growth chambers [16 hr illumination (fluorescent + incandescent)/day, 85% relative humidity, 22°C].

Genotypic analysis

DNA samples from leaf tissue were isolated by a modification of the urea lysis method (Cocciolone and Cone 1993). Genotypes at the *DDM1* locus were deduced by use of a linked CAPS (cleaved amplified polymorphic sequence) (Konieczny and Ausubel 1993) marker, m555 (http://genome-www.stanford.edu/Arabidopsis/aboutcaps.html). A further confirmation of the *ddm1* genotype was made by scoring the methylation of *Hpa*II sites within the centromeric 180-bp repeats and major rDNA repeat as described in Vongs et al. (1993).

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Protoplasts from lines: Parental, B.1, B.2 (Fig. 2), *REVpart*, and *REV2* were harvested from axenically grown seedlings using the method of Doelling and Pikaard (1993). The protoplasting solution was removed by three washes in sorting buffer [0.4 M mannitol, 3 mM MES, 0.1 M KCl, 0.01 M $CaCl_2$, penicillin (50 µg/ml), and streptomycin (25 µg/ml) at pH 5.7 (KOH)]. FACS was preformed using a Becton-Dickinson (San Jose, CA) FACS Vantage machine. Data were collected and processed using CellQuest software for the Macintosh. Excitation was with a broad range long-wave UV light source (330–395 nm), and emission was monitored on the FL4-H channel using a dichroic 405-nm filter cube (395–450 nm). More than 10^6 events were monitored for each of the genotypes.

Fluorometric detection of anthranilate compounds

Leaf samples were ground in 400 µl of ethyl acetate (EtOAc) (J.T. Baker, cat. no. 9280-1) in 1.5-ml microcentrifuge tubes using a micropestle driven by a cordless screwdriver. The samples were spun at 14,000 rpm for 6 min at room temperature in a microcentrifuge, and the supernatant was added to 1.6 ml of EtOAc. The amount of emitted fluorescence was measured using a SPEX FluoroMax spectrofluorometer and SPEX dM3000 software. The excitation wavelength was 340 nm, and the emission spectra were scanned from 360 to 700 nm in 3-nm increments. The intensity at 400 nm (anthranilate compounds) and 680 nm (chlorophyll) was recorded and a ratio calculated to normalize the extraction efficiencies.

Southern blot analysis

Genomic DNA samples were purified using Qiagen protocols and columns, or by the urea lysis miniprep protocol (Cocciolone and Cone 1993) (sector experiment, Fig. 6D). One to two micrograms of genomic DNA was digested with the indicated enzymes (New England Biolabs) using the manufacturer's suggested conditions except that 1 mM spermidine was added to all digestions. Digestion products were separated on 0.8% Sea Kem (FMC) agarose gels, and visualized by ethidium fluorescence. The DNA was blotted to Nytran (Schleicher & Schuell) filters using the Turboblotter (Schleicher & Schuell) system of downward alkaline transfer. Following transfer, the filters were neutralized and the DNA was covalently linked to the filter by UV exposure. Radiolabeled probes were prepared by the random priming method (Ausubel et al. 1987). Hybridizations were done following the protocol of Church and Gilbert (1984). Filters were washed at 65°C in 0.2× SSC, 0.1% SDS. Detection of the radiolabeled probes was done by autoradiography. Quantitation of digestion products was done by phosphorimaging using a Molecular Dynamics PhosphorImager and IPLab gel H version 1.5c (Signal Analytics) software. The *Msp*I–*Hpa*II maps of *PAI2* and *PAI3* were described previously (Bender and Fink 1995). The *Pst*I map was derived from available genomic sequence of *PAI2* or restriction analysis of *PAI3* genomic clones (J. Bender, unpubl.). *Msp*I and *Hpa*II are differentially sensitive to methylation at the cytosines in the 5′-CCGG-3′ recognition sequence (McClelland et al. 1994; Jeddeloh and Richards 1996). *Pst*I is sensitive to methylation of either cytosine in the 5′-CTGCAG-3′ sequence (McClelland et al. 1994).

Genomic sequencing of methylation patterns

For sodium bisulfite mutagenesis, 10 µg of genomic DNA was cleaved with *Xho*I, phenol extracted, and precipitated. The cleaved DNA was alkali denatured in a 235 µl volume of 0.1 M NaOH and 1 mM EDTA at 22°C, neutralized with 50 µl of 1 M Tris-HCl (pH 7.2), and precipitated. Denatured DNA was incubated in the dark at 50 °C for 24 hr in a total volume of 1.2 ml of a freshly prepared solution of 3.2 M sodium bisulfite/0.5 mM hydroquinone (pH 5.0). DNA was recovered from this solution by adding 20 µl of GeneClean (Bio 101) glass milk and processing as specified by the manufacturer. DNA was then incubated for 10 min in 0.3 M NaOH, precipitated, and dissolved in 100 µl of TE (pH 8.0) buffer. PCR reactions were carried out with standard reagents in a 100-µl volume using 1 µl of mutagenized DNA as a template. Products were amplified by cycling 40 times: 1 min at 94°C denaturation, 1 min at 52°C annealing, and 1 min at 72°C extension.

A total of 436 bp from the bottom strand of the *PAI2* promoter region was amplified from mutagenized DNA with the primers P2BF (5′-GGAATTCTTTCTTTTCTAACCAAC-3′) and P2BR (5′-GCTCTAGAGGAAATYTYAGATGGTATYGG-3′). Individual *PAI2* PCR products were subcloned into pBlueScript KSII+ (Stratagene) using the *Eco*RI and *Xba*I sites included in the ends of the primers and sequenced with the T7 primer. As a control to ensure that bisulfite mutagenesis was complete, a region of the *Arabidopsis* genome that is not methylated, 336 bp from the middle of the *ASA1* gene, was amplified with the primers A1BF (5′-GGAATTCACCAACCAAATCTCCTTCC-3′) and

A1BR (5′-GCTCTAGATAGYAAGAAYAATAGGAAGAG-3′). Individual *ASA1* PCR products were subcloned into pBlueScript KSII+ using the *Eco*RI and *Xba*I sites included in the ends of the primers, and four clones were sequenced with the T7 primer. All four of these clones showed complete conversion of cytosines to thymidines. Moreover, 10 other *ASA1* PCR product clones tested had lost an internal *Sac*I site, indicating that they too had undergone mutagenesis. We also observed complete mutagenesis in the upstream 160 bp of every sequenced *PAI2* PCR product. Most of this region is not included in Figure 7.

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The DNA methylation locus*DDM1* is required for maintenance of gene silencing in *Arabidopsis*

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