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Two *Arabidopsis* methylation-deficiency mutations confer only partial effects on a methylated endogenous gene family

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ABSTRACT

In *Arabidopsis* a SWI2/SNF2 chromatin remodeling factor-related protein DDM1 and a cytosine methyltransferase MET1 are required for maintenance of genomic cytosine methylation. Mutations in either gene cause global demethylation. In this work we have assessed the effects of these mutations on the *PAI* tryptophan biosynthetic gene family, which consists of four densely methylated genes arranged as a tail-to-tail inverted repeat plus two unlinked singlet genes. The methylation mutations caused only partial demethylation of the *PAI* loci: *ddm1* had a strong effect on the singlet genes but a weaker effect on the inverted repeat, whereas *met1* had a stronger effect on the inverted repeat than on the singlet genes. The double *ddm1 met1* mutant also displayed partial demethylation of the *PAI* genes, with a pattern similar to the *ddm1* single mutant. To determine the relationship between partial methylation and expression for the singlet *PAI2* gene we constructed a novel reporter strain of *Arabidopsis* in which *PAI2* silencing could be monitored by a blue fluorescent plant phenotype diagnostic of tryptophan pathway defects. This reporter strain revealed that intermediate levels of methylation correlate with intermediate suppression of the fluorescent phenotype.

INTRODUCTION

Many eukaryotes, including mammals and higher plants, modify their genomic DNA by methylation of cytosine residues at the 5 position. The bulk of methylation is found on repetitive sequences, including centromere-associated repeats, *rDNA* arrays and transposon- or retrotransposon-derived repeats (1,2). However, some methylation is also found in single or low copy regions of the genome. Methylation is associated with suppressed gene expression and suppressed homologous recombination and is thought to have evolved as a defense mechanism against invasive repetitive sequences and/or as a means of regulating gene expression.

Mutational analysis of methylation genes in eukaryotes indicates that multiple methylation systems with different specificities contribute to the overall methylation pattern of the genome. For example, mouse ES cells deleted for the cytosine methyltransferase gene *Dnmt1*, *Dnmt3a* or *Dnmt3b* display distinct methylation changes (3,4). In the higher plant model system *Arabidopsis thaliana*, two complementation groups of recessive mutants strongly deficient for methylation of repetitive sequences, *ddm1* and *ddm2*, were isolated by a Southern blot methylation assay (5; E.Richards, Washington University, St Louis, personal communication). The *DDM1* locus encodes a SWI2/SNF2-related protein and might therefore act by influencing the chromatin structure in methylated regions of the genome (6). The *DDM2* locus encodes a cytosine methyltransferase gene *MET1* (E.Richards, personal communication), which has sequence identity to the mouse and human *Dnmt1* methyltransferases (2,7). The *MET1* gene has also been implicated in methylation of repetitive sequences by reverse genetic approaches with antisense transgenes (8,9). Both DDM1- and MET1-deficient strains are viable and fertile, but accumulate a number of morphological defects upon inbreeding, including floral homeotic changes that lead to partial or complete sterility, delayed time to flowering, alteration of leaf shape and reduced size (8–11). These progressive phenotypic changes are likely to be caused, either directly or indirectly, by progressive changes in methylation patterns. In addition, certain floral homeotic changes in *ddm1*, *met1* and antisense *MET1* strains have been shown to be due to local hypermethylation of the *SUPERMAN* gene (12). Hypermethylation of selected genomic regions suggests that DDM1- and MET1-deficient strains retain a *de novo* methylation system.

In this work we have assessed the effects of the *ddm1* and *met1* mutations on the methylated endogenous *PAI* gene family, which encodes the tryptophan biosynthetic enzyme phosphoribosylanthranilate isomerase. In most strains, such as the commonly used laboratory strain Columbia (Col), the *PAI* gene family consists of three unlinked singlet genes (*PAI1*, *PAI2* and *PAI3*) that are completely unmethylated. In contrast, in a few strains, such as Wassilewskija (WS), the *PAI* gene family consists of a tandem inverted repeat duplication of two genes (*PAI1-PAI4*) and two unlinked singlet genes (*PAI2* and *PAI3*) and all four genes are densely cytosine methylated at both 5′-CG-3′ (CG) and non-5′-CG-3′ (non-CG) cytosines (13–15; Figs 1 and 2 and Table 1). No instability or variability

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Table 1. Effects of methylation mutations on patterns of *PAI* promoter cytosine methylation[a]

Strain	*PAI* gene	$C^{Me}G$	$C^{Me}NG$	Other C^{Me}	Total C^{Me}
WS	*PAI1*	236 (100%)	127 (100%)	298 (100%)	661 (100%)
WS*ddm1*	*PAI1*	201 (85%)	104 (81%)	176 (60%)	481 (73%)
WS*met1*	*PAI1*	61 (26%)	52 (41%)	142 (48%)	255 (39%)
WS	*PAI2*	245 (100%)	112 (100%)	341 (100%)	698 (100%)
WS*ddm1*	*PAI2*	123 (50%)	27 (24%)	76 (22%)	226 (32%)
WS *met1*	*PAI2*	131 (53%)	44 (39%)	136 (40%)	311 (45%)

[a]Numbers represent the indicated type of methylated cytosines for eight independent top strand plus eight independent bottom strand bisulfite sequencing clones for the indicated *PAI* promoter region of the indicated strain. The same data are shown in diagram form in Figure 2.

in this dense methylation has been detected in WS tissues grown under standard laboratory conditions. The WS strain is phenotypically normal due to expression of the *PAI1* gene in the inverted repeat (14). This expression might be due to the presence of duplicated promoter sequences from another gene fused in an upstream unmethylated region to the *PAI1*-proximal methylated promoter sequences. The *PAI3* and *PAI4* genes do not encode functional enzyme and are not expressed and the *PAI2* gene, although functional, is silenced by cytosine methylation.

The WS *PAI1-PAI4* inverted repeat locus can trigger *de novo* methylation of an unmethylated *PAI2* gene crossed in from a different strain background (15). Moreover, when the *PAI1-PAI4* inverted repeat is removed from the genome, either by deletion mutation or by segregation in crosses with a strain that carries a singlet *PAI1* gene at this locus, methylation on the *PAI2* gene is reduced in density and persists mainly at CG cytosines (13,15,16). Thus, the *PAI1-PAI4* locus provides a signal for the dense methylation of *PAI* sequences.

In previous work we found that the *ddm1* mutation can strongly hypomethylate the CG-methylated *PAI2* gene in a Δ*pai1-pai4* strain background after a few generations of inbreeding (16). In this work we show that the *ddm1* and *met1* mutations, either singly or combined, reduce but do not eliminate *PAI* methylation when crossed into the WS strain background. These results suggest that the presence of the *PAI1-PAI4* inverted repeat opposes the effects of the methylation mutations. We also describe a novel reporter strain derived from WS where the dense methylation and silencing of the *PAI2* gene can be visualized by tryptophan deficiency phenotypes, including blue fluorescence under UV light due to accumulation of early tryptophan biosynthetic intermediates. Using this reporter strain we show that *ddm1* and *met1* mutations can partially relieve *PAI2* silencing, consistent with their partial effects on *PAI2* methylation.

MATERIALS AND METHODS

Plant strain construction

The WS *ddm1* strain was made by crossing the Columbia *ddm1-2* allele with wild-type WS and genotyping F_2 progeny plants with polymorphic markers at *PAI1*, *PAI2*, *PAI3* and m555, which lies ~1 cM away from the *DDM1* locus (http://www.arabidopsis.org/aboutcaps.html). All *ddm1* single mutant genotypes were confirmed by PCR amplification of the gene with a primer pair that creates a restriction site for *Rsa*I just beyond the end of one primer on a wild-type *DDM1* template but not on a *ddm1-2* mutant template due to the presence of the *ddm1-2* mutation (DDM1-2F, 5′-GTTGGACAGTGTG-GTAAATTCCGCT-3′; DDM1-2R, 5′-GAGCTACGAGCCA-TGGGTTTGTGAAACGTA-3′; where the underlined base represents a mismatch with the *DDM1* gene that creates an *Rsa*I site). The WS *met1* strain was made by crossing the *ddm2-1* allele, which was originally isolated in the Columbia background and then introgressed by crossing three times into the Landsberg *erecta* strain background, with wild-type WS. F_2 progeny plants were genotyped with the *PAI* gene polymorphic markers and with a polymorphism created by the *ddm2-1* mutation. Specifically, primers that flank the *ddm2-1* mutation, MTGF (5′-CCGGTGCATATGGAGTATCCC-3′) and MTGR (5′-GCATCGAATGAGGTTAAGCTC-3′), were used to PCR amplify this region from plant genomic DNA and the PCR products were cleaved with *Hae*III, which cuts once in the wild-type product but fails to cleave in the mutant product due to the *ddm2-1* base change. For both WS *ddm1* and WS *met1* four independent lines were followed for methylation changes by Southern blot assay for four generations of inbreeding and no significant differences among lines were detected. One representative line of each construct was selected for additional inbreeding, bisulfite genomic sequencing and crossing to generate the WS *ddm1 met1* double mutant lines.

For double mutant construction, several hundred F_2 progeny from a cross of WS *ddm1* × WS *met1* were grown in soil until the flowering stage and individuals displaying floral abnormalities and sterility were discarded. The remaining 86 fertile plants were scored for *ddm1* and *met1* genotypes with the m555 and MTG markers, which both lie on the lower arm of chromosome 5. This analysis yielded 19 plants that were homozygous for *ddm1* but heterozygous for *met1*, eight plants that were homozygous for *met1* but heterozygous for *ddm1* and no plants that were double homozygous mutants. To generate double homozygous mutants one *ddm1* homozygous *met1* heterozygous fertile F_2 individual and one *ddm1* heterozygous *met1* homozygous fertile F_2 individual were allowed to self-pollinate and the segregating F_3 progeny were scored for *ddm1* and *met1* genotypes. Double homozygous progeny from both the '*ddm1* first, *met1* second' and the '*met1* first, *ddm1* second' lineages were inbred for one generation and DNA was

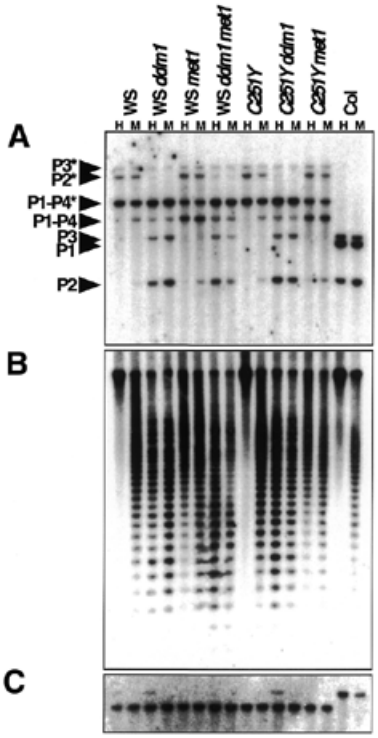

Figure 1. (**A**) *PAI* internal methylation patterns in methylation mutant strains. A genomic Southern blot of DNA prepared from whole 4-week-old plants of the indicated strains cleaved with either *Hpa*II (H) or *Msp*I (M) and probed with an internal *PAI* probe is shown. The WS *ddm1* and WS *met1* DNAs were extracted from a representative line of each strain inbred for six generations in the presence of the homozygous mutation. The WS *ddm1 met1* DNA was extracted from a representative line that was inbred for one generation in the presence of both homozygous mutations. The *C251Y* (WS *pai1C251Y*) *ddm1* and *C251Y met1* DNAs were extracted from a representative line of each strain inbred for two generations in the presence of the homozygous mutation. The *Msp*I restriction maps of the WS and Col *PAI* genes were previously described (13–15). The band sizes predicted for the *PAI* loci (P1-P4 is WS *PAI1-PAI4*, P1 is Col *PAI1*, P2 is WS or Col *PAI2*, P3 is WS or Col *PAI3*) are indicated in the left margin, with the fully cleaved species unmarked and the internally methylated species marked with an asterisk. (**B**) Centromere repeat methylation patterns. The same blot shown in (A) was stripped and reprobed with a fragment that detects the 180 bp centromere repeats. (**C**) Digestion pattern of the *ASA1* gene. The same blot shown in (A) was stripped and reprobed with an *ASA1* (anthranilate synthase α subunit 1) cDNA probe. Because the *ASA1* region carries very little methylation, *Hpa*II and *Msp*I give similar restriction patterns. The probe detects a 4.9 kb band in WS and a 5.9 kb band in Col due to an *Msp*I polymorphism. The *ASA1* blot thus controls for equal loading and complete digestion of DNA samples.

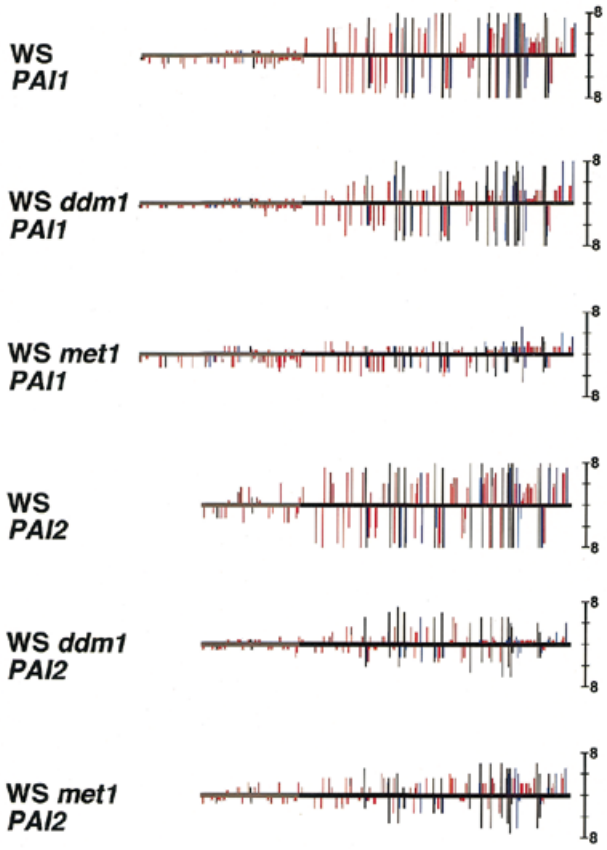

Figure 2. *PAI* promoter methylation patterns in methylation mutant strains. Bisulfite genomic sequencing of methylation patterns was performed for the top and bottom strands of the indicated *PAI* gene promoters in a representative line of four generation inbred WS *ddm1* and a representative line of four generation inbred WS *met1*, with eight independent molecules sequenced for each strand. Vertical lines indicate the positions of cytosines, with the height of each line representing how many sequenced molecules had 5-methylcytosine (5-MeC) at that position. Black indicates cytosines in the context CG, blue indicates cytosines in the context CNG and red indicates cytosines in other contexts. Asterisks indicate sites where none of the sequenced molecules had a 5-MeC. The black horizontal line indicates the region of *PAI* identity and the gray horizontal line indicates flanking upstream heterologous sequence unique to each gene. For the purposes of comparison our previously published sequencing data for the wild-type WS *PAI1* and *PAI2* promoter regions are also shown; these data are reprinted from Luff *et al.* (15) with permission from Elsevier Science.

prepared from second generation progeny plants for Southern blot analysis. *PAI* and centromere methylation patterns were identical for both types of lineages. DNA from the '*met1* first, *ddm1* second' lineage is shown in Figure 1.

The WS *pai1C251Y* strain was made by EMS mutagenesis of WS using standard methods (17), followed by screening ~70 000 M_2 progeny plants for blue fluorescence as described (13). Fluorescent isolates caused by spontaneous deletion of the *PAI1-PAI4* inverted repeat (13) were identified by DNA analysis and discarded. The remaining fluorescent isolates were tested by complementation crosses with the WS Δ*pai1-pai4* fluorescent mutant. The WS *pai1C251Y* allele was further characterized by cloning and sequencing the *PAI1* gene. The strain was backcrossed twice to wild-type WS before performing methylation and double mutant analysis. Another *pai1* missense mutation isolated from the screen, *pai1G226E*, had more severe growth and fertility defects than *pai1C251Y* and therefore was not pursued as a reporter strain.

Seedlings were germinated on plant nutrient plus sucrose medium (18) under continuous illumination. Adult plants were grown in Scotts MetroMix 360 potting medium under continuous illumination.

DNA and RNA analysis

Plant genomic DNA for Southern blot and bisulfite genomic sequencing methylation analysis and plant RNA for northern blot analysis were prepared as previously described (14). The *PAI* probe is a 0.7 kb internal *Pst*I fragment isolated from a Col *PAI1* cDNA (13). The centromere 180 bp repeat probe was isolated as a *Hin*dIII fragment from plasmid pARR20-1 (a gift

of E.Richards). Bisulfite genomic sequencing was performed as previously described (16). Briefly, 10 µg DNA was cleaved with *Xho*I to separate the two halves of the *PAI1-PAI4* inverted repeat, mutagenized with sodium bisulfite and tested for efficiency of mutagenesis by analysis of the unmethylated *ASA1* gene. Eight independent PCR products for each region (*PAI1* top strand, *PAI1* bottom strand, *PAI2* top strand, *PAI2* bottom strand) were cloned into pBlueScript KS II+ (Stratagene) for sequencing. Primer sequences for the *PAI2* bottom strand were as previously described (16) and primer sequences for the other amplified regions are available upon request from J.Bender.

RESULTS

PAI genes are partially hypomethylated in WS *ddm1* and WS *met1* strains

The *Arabidopsis* hypomethylation mutations *ddm1* and *met1* were isolated in the Col strain background, which lacks a *PAI* inverted repeat gene structure and *PAI* methylation (13,14). The mutations were therefore crossed into the WS background to assess effects on *PAI* methylation. Polymorphisms associated with each locus were used to identify plants that were homozygous for the relevant methylation mutation from Col and the *PAI1-PAI4*, *PAI2* and *PAI3* loci from WS (WS *ddm1* and WS *met1*). The *ddm1* allele *ddm1-2* creates a splice site mutation that results in misprocessing of the *DDM1* transcript and is likely to be a null allele (6). The *met1* allele *ddm2-1* is a missense mutation in an unconserved position of the cytosine methyltransferase catalytic domain (E.Richards, personal communication).

PAI methylation in WS *ddm1* and WS *met1* strains was monitored by a *Hpa*II/*Msp*I Southern blot assay over six generations of inbreeding. The restriction enzymes *Hpa*II and *Msp*I are isoschizomers that recognize the sequence 5′-CCGG-3′; *Hpa*II is sensitive to methylation of either the inner or the outer cytosine of the sequence, whereas *Msp*I is sensitive only to methylation of the outer cytosine of the sequence. Each *PAI* locus is cleaved once at a conserved *Hpa*II/*Msp*I site in the second intron and at flanking unmethylated sites that lie at different distances away from the central site for each locus (13–15). Thus, each *PAI* locus gives unique fragment sizes both when fully cleaved (diagnostic of no *PAI* methylation) or when uncleaved (diagnostic of *PAI* methylation). Previous analyses showed that the *Hpa*II/*Msp*I methylation correlates with methylation patterns over the rest of the *PAI* sequences for wild-type WS (13,15).

The *Hpa*II/*Msp*I assay revealed that the *ddm1* mutation had only a weak effect on methylation of the *PAI1-PAI4* inverted repeat locus, but had a strong hypomethylation effect on the singlet *PAI2* and *PAI3* genes (Fig. 1A). This basic pattern was established by the second generation, although the *PAI2* and *PAI3* genes became progressively less methylated over four subsequent generations of inbreeding. The *met1* mutation had an intermediate hypomethylation effect on the inverted repeat *PAI1-PAI4* locus, but had a weaker effect on the *PAI2* and *PAI3* genes. Note that because the *PAI3* gene is divergent in sequence from the probe fragment (13,16), this locus is not as strongly detected as *PAI1-PAI4* and *PAI2* in hybridization experiments. Thus, the hypomethylated *PAI3* species was only detectable on overexposure of the Figure 1A blot (data not shown). The *PAI* methylation pattern shown in Figure 1A remained constant over the entire WS *met1* inbreeding regime monitored from the second generation onwards.

Both WS *ddm1* and WS *met1* inbred lines progressively accumulated a number of morphological defects and reduced fertility, as previously observed in the Col strain background (9–11). In particular, the most inbred WS *ddm1* line developed flowers with unfused carpels and the most inbred WS *met1* line was late flowering and displayed a number of floral abnormalities (data not shown).

We also crossed an antisense *MET1* transgene (8) into the WS strain background and monitored effects on *PAI* methylation by Southern blot assay. The antisense *MET1* transgene had a similar effect to the *met1* missense mutation on WS *PAI* gene methylation in second generation DNA (data not shown). Because our antisense transgene-carrying lines became completely sterile within two generations of inbreeding we did not pursue analysis of these lines.

We also constructed a *ddm1 met1* double mutant in the WS strain background (WS *ddm1 met1*) by crossing together WS *ddm1* and WS *met1* lines and using polymorphisms associated with the methylation mutations to identify double mutant recombinants. A majority of plants in the segregating population from this cross were late flowering and/or sterile, presumably due to accumulation of methylation changes during the long inbreeding regime of the parental strains (see above). However, we were able to recover two independent double mutant individuals that were fertile when newly segregated. The double mutants had a number of morphological defects and became completely sterile by the second generation. We prepared DNA from the second generation progeny of each double mutant lineage and analyzed *PAI* methylation by Southern blot. This analysis showed that the WS *ddm1 met1* double mutants displayed strong hypomethylation of *PAI2* and *PAI3* but weak hypomethylation of the *PAI1-PAI4* locus, similarly to the *ddm1* single mutant (Fig. 1A). Thus, the combined methylation mutations were not sufficient to remove *PAI* methylation after two generations of inbreeding.

The 180 bp centromere-associated repeats were strongly hypomethylated in the WS methylation mutant hybrid strains (Fig. 1B), consistent with previous analyses in the Col and C24 strain backgrounds (5,8,9). The centromere repeat hypomethylation was stronger in the *ddm1* and *ddm1 met1* mutant backgrounds than in the *met1* mutant background, as estimated from the pattern of cleavage with *Hpa*II.

The *ddm1* and *met1* mutations reduce both symmetrical and asymmetrical *PAI* methylation

To understand the effects of *ddm1* and *met1* mutations on *PAI* methylation at the sequence level we performed sodium bisulfite genomic sequencing on the promoter regions of the *PAI1* inverted repeat gene and the *PAI2* singlet gene in genomic DNA prepared from four generation inbred plants. In this method genomic DNA is treated with sodium bisulfite under conditions where unmethylated cytosines are deaminated to uracil but methylated cytosines remain unreacted (19). The genomic region of interest is then amplified by PCR and C→T mutations in the PCR products indicate the positions of unmethylated residues.

In WS *ddm1* we found that the methylation patterns for the *PAI1* promoter at the inverted repeat locus were similar to those previously determined by sequencing the same region in parental WS (15): within the region of *PAI* sequence identity symmetrical CG and CNG as well as asymmetrical cytosines were methylated and there was no significant spread of methylation into upstream heterologous sequences (Fig. 2). The primary difference between WS and WS *ddm1 PAI1* methylation patterns was that the WS *ddm1* methylation density was 73% of that found in WS (Table 1). In contrast, for the singlet *PAI2* gene in WS *ddm1* methylation was reduced to 32% of parental WS levels. Note that this degree of hypomethylation is likely to be an underestimate of the ultimate effect of *ddm1* on *PAI2*, because *Hpa*II/*Msp*I Southern blot analysis revealed a progressive loss of *PAI2* methylation between the fourth generation of inbreeding, when the sequencing was performed, and the sixth generation of inbreeding (shown in Fig. 1A). For both *PAI1* and *PAI2* sequences the *ddm1* mutation reduced both symmetrical (CG and CNG) and asymmetrical cytosine methylation, although there was a stronger effect on non-CG methylation (Table 1).

In WS *met1* both the *PAI1* and *PAI2* promoters had <50% residual methylation relative to parental WS (Fig. 2 and Table 1). As observed for *ddm1*, methylation of both symmetrical and asymmetrical cytosines was affected by the *met1* mutation. We were unable to perform *PAI* bisulfite sequencing on the WS *ddm1 met1* and WS antisense *MET1* strains due to the low amounts of DNA recovered from these morphologically aberrant and sterile strains.

The bisulfite sequencing patterns were generally consistent with the WS *ddm1 Hpa*II/*Msp*I digestion patterns, showing weak hypomethylation at the *PAI1-PAI4* inverted repeat locus and stronger hypomethylation at the singlet *PAI2* locus, and with the WS *met1* patterns, showing partially reduced methylation for both *PAI1-PAI4* and *PAI2* (Fig. 1A). However, the bisulfite sequencing revealed a stronger effect of *met1* on the *PAI2* gene, with ~45% residual methylation, than was indicated by the Southern blot analysis. This discrepancy could reflect a different effect of the *met1* mutation near the methylation boundary in the sequenced promoter region versus the internal *Hpa*II/*Msp*I site.

PAI steady-state transcript levels are not dramatically altered by *ddm1* or *met1* mutations

In wild-type WS the *PAI1* transcript is the predominant species detected by northern blot, cDNA analysis or RT–PCR approaches and provides sufficient PAI enzyme so that WS is a phenotypically normal strain (13,14). We used the WS *ddm1* and WS *met1* strains to determine whether this *PAI* expression profile could be significantly altered by reduced methylation. From previous studies in strains that lack *PAI1-PAI4* we knew that *PAI3* has very low expression regardless of methylation state (14) and that *PAI2* can be reactivated up to 30% of the WS *PAI1* steady-state transcript levels by strong demethylation (13). However, we did not know whether *PAI1* or *PAI4* expression levels would be affected by methylation changes.

We monitored *PAI* expression levels in WS methylation mutant strains versus parental WS by northern blot analysis of 4-week-old whole plant RNA with a *PAI1* cDNA probe which can detect all of the highly identical *PAI* sequences (Fig. 3). In wild-type WS the signal detected by this probe is >90% *PAI1* transcripts (14). The signal level was only slightly increased in the methylation mutant strains, indicating that overall *PAI* expression is not dramatically altered by reduced methylation. In order to determine which *PAI* genes might contribute to the slight changes in expression we performed RT–PCR reactions on mutant RNA templates with primer pairs that discriminate *PAI1* and *PAI4* transcripts from *PAI2* and *PAI3* transcripts via a restriction site polymorphism or that discriminate *PAI4* transcripts from other *PAI* transcripts via a length polymorphism (14). We were only able to detect *PAI1* species by these assays (data not shown). However, based on control reactions, we estimate that the RT–PCR primers can only reliably detect *PAI* transcript species that are present at ≥10% of the template population. Thus, it was inconclusive as to which *PAI* genes might be slightly up-regulated by partial demethylation using these assays.

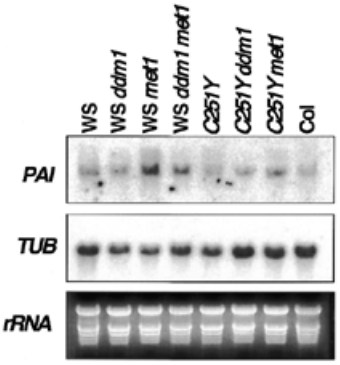

Figure 3. *PAI* steady-state transcript levels are not dramatically perturbed by methylation mutation-induced hypomethylation. Total RNA was prepared from 4-week-old plants of the same strains described in Figure 1. Duplicate northern blots prepared from these samples were probed with either the *PAI* cDNA 0.7 kb internal *Pst*I fragment (3 day exposure) or with a β-tubulin probe (*TUB*) as a control for gel loading (16 h exposure). The ethidium bromide stained gel of the *PAI* northern blot (*rRNA*) is also shown as a control for gel loading.

Hypomethylation by the *ddm1* and *met1* mutations activates *PAI2* expression

Previous northern blot analysis of WS *PAI2* expression in a strain background where the *PAI1-PAI4* genes are deleted and where the residual methylation of *PAI2* is reduced and destabilized indicated that the partially methylated *PAI2* gene has only ~10% of the steady-state *PAI* transcript levels observed for parental WS (13). Thus, increased expression from a partially methylated *PAI2* gene in methylation mutant lines would not be detected by northern blot or RT–PCR over the strong signal from *PAI1*. As a more sensitive means of following WS *PAI2* expression we isolated a derivative of WS that displayed a number of PAI-deficient phenotypes due to a missense mutation in the *PAI1* gene. In this strain background changes in *PAI* expression are easily monitored as changes in *pai* mutant phenotypes.

The *pai1* missense mutant reporter strain WS *pai1C251Y* was strongly blue fluorescent under UV light due to the accumulation of early intermediates in the tryptophan pathway (20,21) and had yellow-green leaf pigmentation, reduced size and increased bushiness relative to parental WS (Fig. 4). However, the strain was viable and fertile even on unsupplemented agar medium, indicating that there was sufficient residual PAI

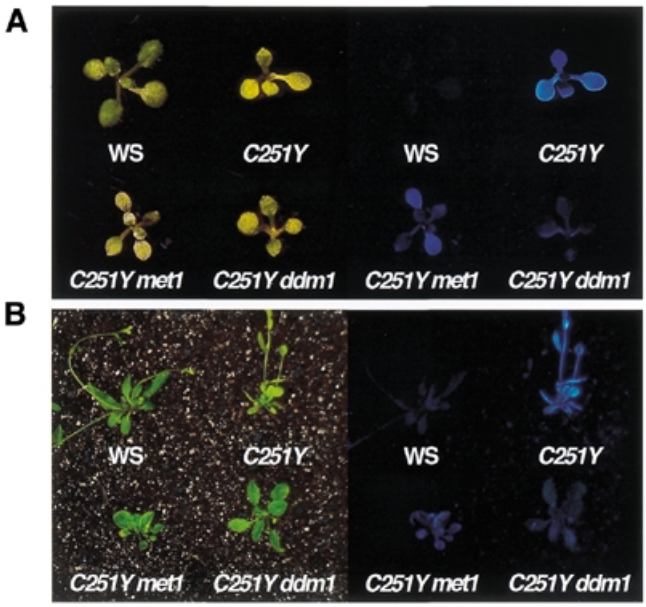

Figure 4. The methylation mutants suppress *PAI*-deficient phenotypes of the WS *pai1C251Y* reporter strain. (**A**) Representative 2-week-old seedlings of the indicated genotypes photographed under visible (left) and UV (right) light are shown. (**B**) Representative 4-week-old plants of the indicated genotypes photographed under visible (left) and UV (right) light are shown. The *C251Y ddm1* and *C251Y met1* plants are from the F_5 generation, the same generation used to prepare DNA and RNA for molecular analysis (Figs 1 and 3).

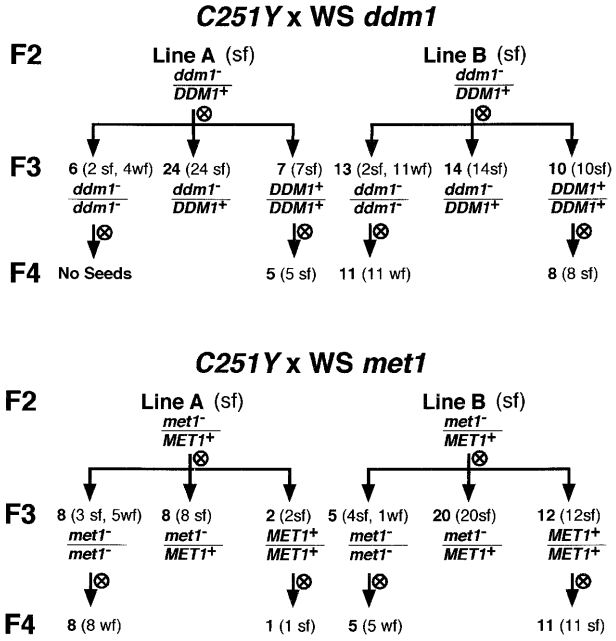

Figure 5. Genetic pedigrees used to analyze the effects of *ddm1* and *met1* on the WS *pai1C251Y* reporter strain. The circled X symbol indicates self-pollination. In the F_4 generation phenotypes were assigned based on the average behavior of a population of 24 plants. sf indicates a strong fluorescent phenotype equivalent to that of the parental *pai1C251Y* strain. wf indicates a weaker fluorescent phenotype than displayed by the parental *pai1C251Y* strain.

enzyme produced from the mutant *pai1* gene and/or the silenced *PAI2* gene, so that the strain was not a tryptophan auxotroph. Neither *PAI* methylation nor *PAI* gene expression were significantly affected by the point mutation in the WS *pai1C251Y* strain (Figs 1 and 3). The strong blue fluorescence phenotype of the mutant was completely stable in thousands of plants analyzed over several generations of inbreeding, indicating that WS *pai1C251Y* does not segregate progeny with spontaneously hypomethylated and activated *PAI* genes at a significant frequency.

To monitor effects of *ddm*-induced hypomethylation on *PAI* expression we crossed the WS *pai1C251Y* fluorescent reporter strain with WS *ddm1* or WS *met1* and used the fluorescence phenotype plus polymorphic markers at each methylation mutation to identify double mutant plants. Specifically, to assess the effects of the methylation mutations in the *pai1C251Y* background without the complication of lingering epigenetic changes inherited from the *ddm1* and *met1* parents, we identified two F_2 individuals from each cross that were phenotypically identical to the parental *pai1C251Y* mutant and heterozygous for the relevant methylation mutation. We then analyzed segregation of fluorescence phenotype versus *ddm1* or *met1* genotype in a sampling of F_3 progeny of each cross (Fig. 5).

For the *pai1C251Y ddm1* cross we found that newly segregated double mutant F_3 individuals had phenotypes ranging from strong fluorescence and morphology equivalent to that of the parental *pai1C251Y* mutant to partial fluorescence with increased plant size and fertility (Fig. 5). In contrast, for the *pai1C251Y met1* cross all of the newly segregated double mutant F_3 individuals had a strong fluorescent phenotype. These results suggest that the *ddm1* mutation has a variable partial effect on *PAI* expression and that *met1* has little or no effect when newly segregated.

To assess effects of the methylation mutations in the second generation of homozygosity, 24 F_4 progeny for each of a number of representative F_3 double mutant individuals were scored for fluorescence and morphology relative to the parental WS *pai1C251Y* strain (Fig. 5). For *pai1C251Y ddm1* all the members of each F_4 family displayed strongly suppressed *pai* phenotypes, including extremely weak fluorescence and increased size and fertility (Fig. 4), regardless of the suppressed phenotype of the parental F_3 plant. For *pai1C251Y met1* all the members of each F_4 family displayed partially suppressed fluorescence with increased size and fertility. These results indicate that the *ddm1* and *met1* mutations can progressively suppress the PAI deficiencies of the WS *pai1C251Y* strain and that after one generation of inbreeding there is uniform suppression. These results also suggest that *ddm1* has a stronger effect than *met1*, consistent with the relative effects of the two mutations on *PAI2* methylation density (Figs 1 and 2 and Table 1). Similar progressive effects of the *ddm1* mutation on *PAI2* silencing were previously observed in a derivative of WS where the *PAI1-PAI4* locus is deleted and the *PAI2* gene carries reduced residual methylation (16).

Both the *pai1C251Y ddm1* and the *pai1C251Y met1* lines were inbred for a second generation (F_5 generation) to determine if there were additional progressive effects on the fluorescence phenotype (Fig. 4). Double mutant plants in this generation displayed little or no phenotypic difference from the previous generation, indicating that after the first generation of inbreeding the effects of the methylation mutations on *PAI*

silencing are largely complete. This result is consistent with our observation that methylation patterns as monitored from the first generation of inbreeding onwards in the WS *ddm1* and WS *met1* lines were largely unchanged (see above).

Genomic DNA was prepared from representative WS *pai1C251Y ddm1* or *met1* homozygous double mutant F_5 tissue for methylation analysis of the *PAI* genes. Southern blot assays with *Hpa*II and *Msp*I revealed that the *ddm1* and *met1* mutations had the same effects on methylation in the WS *pai1C251Y* mutant background as when crossed into wild-type WS (Fig. 1A). Northern blot analysis revealed that, as in the WS background, the *ddm1* and *met1* mutations did not dramatically alter the steady-state levels of *PAI* transcripts in the WS *pai1C251Y* background (Fig. 3). Because the changes in *pai* phenotypes correlate with the degree of *PAI2* hypomethylation (stronger in *ddm1* than in *met1*) rather than with the degree of *PAI1* hypomethylation (stronger in *met1* than in *ddm1*) (Figs 1, 2 and 4 and Table 1), it is most likely that the phenotypic changes arise primarily from activation of *PAI2* expression.

DISCUSSION

Studies of the methylation-deficient mutations *ddm1* and *met1* in *Arabidopsis* indicated that these mutations confer strongly reduced methylation of most genomic sequences (5,8–10). In contrast, we found that these mutations, either singly or combined, confer only partial hypomethylation of the methylated *PAI* gene family. A unique feature of the *PAI* family is that two of the genes, *PAI1-PAI4*, are arranged as an inverted repeat. In previous work we showed that the *PAI1-PAI4* locus triggers *de novo* methylation of *PAI* sequences and that removal of this locus from the genome results in reduced methylation on unlinked *PAI* genes (13,15,16). Thus, we favor the view that the *PAI* genes resist hypomethylation by the *ddm1* and *met1* mutations because of an opposing methylation-stimulatory effect from the inverted repeat locus.

The residual partial methylation on the *PAI* genes in the *ddm1* and *met1* strains gave us a unique opportunity to examine patterns of cytosine methylation promoted by other methylation activities. For both mutations we found that CG and non-CG methylation persist, indicating that secondary methylation systems have both types of substrate specificities. To date no other *Arabidopsis* methylation functions have been described based on genetic analysis, but several candidate methyltransferase genes have been identified in the genome sequence, including close homologs of *MET1* (22,23), chromomethylase genes carrying a chromo domain motif in the predicted catalytic region of the protein (22–25) and domain-rearranged methyltransferases with an altered order of catalytic motifs in the primary amino acid sequence and homology to the mammalian *Dnmt3* family (26).

Our analysis showed that each methylation mutation had a different effect on *PAI1-PAI4* inverted repeat versus singlet *PAI* gene methylation (Figs 1 and 6). The *ddm1* mutation, in a chromatin remodeling factor-related protein (6), had a strong effect on singlet gene methylation but a weaker effect on inverted repeat methylation. The *met1* mutation, in a cytosine methyltransferase (E.Richards, personal communication), had a stronger effect on the inverted repeat than on the singlet *PAI* genes. Thus, even though all three wild-type WS *PAI* loci carry uniform dense methylation (15), the three loci are differentially

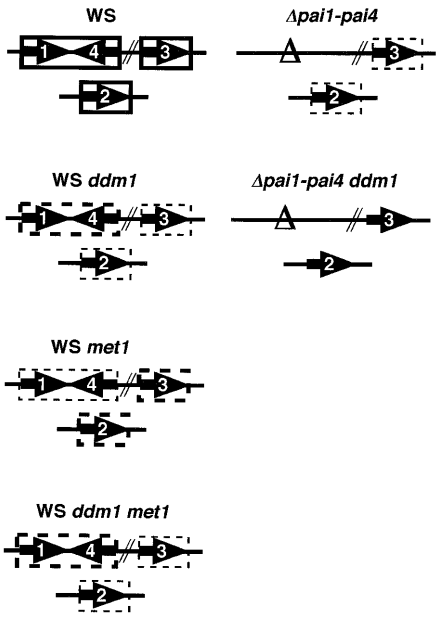

Figure 6. A summary of methylation changes on the *PAI* genes in various mutant backgrounds. The organization of the four *PAI* genes in WS is shown. The arrows depict the direction of transcription for each gene, with the hatch marks between the *PAI1-PAI4* locus and the *PAI3* locus indicating that these loci are on the same chromosome but genetically unlinked. The Δ symbol represents deletion of the *PAI1-PAI4* genes. The boxes around each gene locus indicate cytosine methylation. The approximate density of methylation at each locus is indicated as follows: a solid bold line indicates parental WS levels of methylation, a bold dashed line indicates a <50% reduction in methylation, a fine dashed line indicates a >50% reduction in methylation and no line indicates only trace levels or no detectable methylation. Density indications are based on combined results from *Hpa*II/*Msp*I Southern blot and promoter bisulfite sequencing analyses, except for the WS *ddm1 met1* strain, which is based solely on Southern blot analysis.

demethylated by *ddm1* and *met1*. This observation suggests that methylation determinants for residual methylation systems in each mutant background vary with each *PAI* locus. Methylation determinants are likely to include specific chromatin features unique to each locus that contribute to targeting of methylation proteins and/or accessibility of the substrate DNA. Our analysis also showed that the overall *PAI* methylation patterns conferred by the two mutations are different from each other. This observation suggests that DDM1 and MET1 act at least partially independently.

For two WS *ddm1 met1* double mutant lineages that we examined we found residual *PAI* methylation by Southern blot assay of second generation DNA (Figs 1 and 6). This result indicates that at least some of the residual methylation systems in *Arabidopsis* can act independently of *ddm1* and *met1*. We also found that the pattern of partial methylation in both lineages was similar to that of the *ddm1* single mutant, with a high level of residual methylation on the inverted repeat and much lower levels on the singlet *PAI* genes. This result suggests a complex epistatic relationship between *ddm1* and *met1* rather than a simple additive effect of the two mutations, which would have been expected to yield plants with low residual methylation on all three *PAI* loci. For example, because methylation of the inverted repeat is apparently higher in the *ddm1 met1* double mutant than in the *met1* single mutant, it could be postulated

that a secondary methylation system with specificity for the inverted repeat is selectively activated in the presence of the *ddm1* mutation. However, there are two alternative explanations for the apparently epistatic pattern of double mutant methylation. First, the Southern blot assay, which only monitors one site in each *PAI* locus, might not reflect methylation changes over the rest of the *PAI* sequences. Secondly, it is possible that the two double mutant lineages we examined happened to inherit and maintain *ddm1*-modified *PAI* loci from their *ddm1* parent.

The WS *pai1C251Y* blue fluorescent mutant strain described here provides a facile reporter for methylation and transcriptional silencing of the *PAI2* gene. Using this reporter strain we showed that loss of *PAI2* silencing is progressive for both the *ddm1* and *met1* mutations, so that newly segregated homozygous mutants have stronger fluorescence phenotypes than their first generation progeny plants (Figs 4 and 5). In both cases progressive loss of *PAI2* silencing is likely to reflect impaired but not abolished maintenance methylation activity. For the *ddm1* mutation maintenance methylation might be impaired but not abolished as an indirect consequence of chromatin changes induced by loss of DDM1 function; for the *met1* mutation, maintenance methylation might be impaired but not abolished due to a partial loss-of-function allele and/or due to redundant methyltransferases. The progressive relief of *PAI2* silencing by *ddm1* in the presence of the *PAI1-PAI4* inverted repeat is similar to the effect we previously observed in the Δ*pai1-pai4* background. However, *ddm1* ultimately has a stronger effect in the deletion mutant strain, where it confers almost complete loss of *PAI2* promoter methylation after just two generations of inbreeding (16), compared to 32% residual methylation after four generations of inbreeding (Table 1 and Fig. 6). This difference is likely to reflect the methylation-stimulatory effects of the *PAI1-PAI4* locus acting in opposition to *ddm1*-induced hypomethylation, as discussed above.

In the WS *pai1C251Y* background the density of methylation on *PAI2* (Fig. 1) corresponds to the severity of the PAI-deficient blue fluorescent phenotype (Fig. 4). Specifically, the strong hypomethylation of *PAI2* by *ddm1* is reflected in a weak fluorescent phenotype and the partial hypomethylation of *PAI2* by *met1* is reflected in an intermediate fluorescent phenotype. Beyond the analysis of *ddm1* and *met1* mutations the *pai1C251Y* strain promises to be a powerful tool in explicit analysis of other *Arabidopsis* mutations that are implicated in methylation, chromatin structure and gene silencing. Furthermore, the *pai1C251Y* strain provides a genetic screening system for novel mutations that disrupt *PAI2* methylation and silencing.

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