Arabidopsis *MET1* Cytosine Methyltransferase Mutants

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Manuscript received July 6, 2002
Accepted for publication December 9, 2002

ABSTRACT

We describe the isolation and characterization of two missense mutations in the cytosine-DNA-methyltransferase gene, *MET1*, from the flowering plant *Arabidopsis thaliana*. Both missense mutations, which affect the catalytic domain of the protein, led to a global reduction of cytosine methylation throughout the genome. Surprisingly, the *met1-2* allele, with the weaker DNA hypomethylation phenotype, alters a well-conserved residue in methyltransferase signature motif I. The stronger *met1-1* allele caused late flowering and a heterochronic delay in the juvenile-to-adult rosette leaf transition. The distribution of late-flowering phenotypes in a mapping population segregating *met1-1* indicates that the flowering-time phenotype is caused by the accumulation of inherited defects at loci unlinked to the *met1* mutation. The delay in flowering time is due in part to the formation and inheritance of hypomethylated *fwa* epialleles, but inherited defects at other loci are likely to contribute as well. Centromeric repeat arrays hypomethylated in *met1-1* mutants are partially remethylated when introduced into a wild-type background, in contrast to genomic sequences hypomethylated in *ddm1* mutants. *ddm1 met1* double mutants were constructed to further our understanding of the mechanism of DDM1 action and the interaction between two major genetic loci affecting global cytosine methylation levels in Arabidopsis.

POSTREPLICATIVE methylation of cytosines is a common DNA modification in eukaryotes. Recent evidence indicates that cytosine methylation is an important epigenetic mark that functions in a complex web of interactions with histone modification codes to articulate epigenetic gene expression states (Jenuwein and Allis 2001; Rice and Allis 2001; Richards and Elgin 2002). The cytosine methylation reaction is carried out by a diverse set of cytosine-DNA-methyltransferases (Colot and Rossignol 1999). Traditionally, two different DNA methyltransferase activities are recognized: (1) a "*de novo*" activity that transfers one methyl group to completely unmethylated double-stranded DNA and (2) a "maintenance" activity that methylates cytosines in proximity with methylcytosines on the complementary strand (Holliday and Pugh 1975; Riggs 1975). The concentration of methylation in short symmetrical sequences (*i.e.*, CpG in plants and vertebrates and CpNpG in plants) and the hemi-methylated substrate preference of extractable methyltransferase activities were early indications of a maintenance methylation system that could preserve methylation patterns after DNA replication.

Cytosine methyltransferases can also be categorized on the basis of enzyme structure and similarity of conserved amino acid motifs. Colot and Rossignol (1999) recently differentiated five different groups of DNA methyltransferases on the basis of these criteria, named after prototypic genes/enzymes in each class: Dnmt1 (Bestor *et al.* 1988), pmt1/Dnmt2 (Wilkinson *et al.* 1995), Dnmt3 (Okano *et al.* 1998), chromomethyltransferases (CMT; Henikoff and Comai 1998), and Masc1 (Colot and Rossignol 1999). The mammalian Dnmt3 (Okano *et al.* 1999; Dodge *et al.* 2002; Yokochi and Robertson 2002) and fungal (Ascobolus) Masc1 (Malagnac *et al.* 1997) enzymes have been demonstrated to be *de novo* methyltransferases, while the Dnmt1 family members are thought to function primarily as maintenance methyltransferases (Li *et al.* 1992; Pradhan *et al.* 1999). Cao *et al.* (2000) identified maize (*Zmet3*) and Arabidopsis (*DRM*) genes encoding proteins closely related to Dnmt3 methyltransferases but containing a novel arrangement of the eight diagnostic methyltransferase amino acid motifs.

Organisms can possess representatives of multiple methyltransferase classes. For example, the Arabidopsis genome contains four *Dnmt1*-class and three CMT-class genes, as well as three *Dnmt3*-like *DRM* genes, and a single *Dnmt2*-like gene (http://www.chromdb.org; Finnegan and Kovac 2000). The specialization of these

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enzymes within the plant nucleus is beginning to be understood. Antisense suppression of the Arabidopsis *Dnmt1*-class gene *MET1* caused a reduction of global cytosine methylation levels, particularly at CpG sites (Finnegan *et al.* 1996; Ronemus *et al.* 1996). Recently, several groups demonstrated that mutations affecting chromomethyltransferase genes lead to a reduction in CpNpG methylation (Bartee *et al.* 2001; Lindroth *et al.* 2001; Papa *et al.* 2001). More recently, the Arabidopsis *DRM* genes have been shown to be responsible for *de novo* methylation at CpNpG and asymmetric sites and share overlapping function with the chromomethyltransferases in maintenance methylation at non-CpG sites (Cao and Jacobsen 2002a,b).

Here we report characterization of Arabidopsis plants with point mutations in the *MET1* gene. Our results indicate that the MET1 protein is responsible for maintaining cytosine methylation throughout the Arabidopsis genome. Further, our results demonstrate that loss of *MET1* function leads to a late-flowering defect that is caused by inherited variation independent of the *met1* mutation. Loss of *MET1* function leads to epigenetic alleles of the flowering-time locus *FWA* (Soppe *et al.* 2000), explaining part of the late-flowering defect.

MATERIALS AND METHODS

Arabidopsis mutagenesis and mutant screen: Ethylmethanesulfonate (EMS)-mutagenized seeds (M2 generation) from strain Columbia (Col), marked with the hairless (glabrous) *gl1* mutation, were purchased from Lehle Seeds (Tucson, AZ). M2 seeds from seven M1 parental groups were planted in subdivided 26 × 52-cm flats using a 60% Redi-Earth (Scotts):40% vermiculite mixture. M2 plants were grown under standard glasshouse conditions with supplemental light during the summer of 1994. DNA hypomethylation mutants were identified by loss of *Hpa*II restriction site methylation in the large 180-bp centromere repeat arrays, as described in Vongs *et al.* (1993). Approximately 5000 M2 plants were screened.

Plant material: For most experiments described here, the *met1-1* mutation was backcrossed three or more times to wild-type Columbia parents or introgressed by five backcrosses into the Landsberg erecta (Ler) strain background. The exception was the 5-methylcytosine analysis shown in Figure 2, where nonbackcrossed *met1* material was used. The *met1-2* mutation was backcrossed four times to wild-type Columbia parents for phenotypic analysis; however, nonbackcrossed *met1-2* material was used for cytosine methylation analysis. The *ddm1-2 met1-1* double mutants were constructed as follows: A *DDM1/ddm1-2* (Columbia, backcrossed six times) individual was crossed onto a *MET1/met1-1* (Columbia, backcrossed three times) individual to generate a *ddm1-2 MET1/DDM1 met1-1 trans*-heterozygote. This plant was self-pollinated to generate either *ddm1-2 MET1/ddm1-2 met1-1* or *DDM1 met1-1/ddm1-2 met1-1* plants, which were identified by molecular genotyping (see below). These plants were allowed to self-pollinate and *ddm1-2 met1-1* homozygotes were recovered from both lineages. Single-mutant homozygotes recovered from these populations were used as controls; consequently all mutant material was closely matched and none had been inbred for more than one generation.

Southern blot analysis: Genomic DNA samples were purified from either pooled tissue (Figures 3, 4, 8, and 11) or individuals (Figure 9) using the urea lysis miniprep protocol of Cocciolone and Cone (1993) or using QIAGEN (Chatsworth, CA) protocols and columns. Genomic DNA was digested with the restriction endonucleases using the manufacturer's (New England Biolabs, Beverly, MA) recommendations, except that spermidine was added to a final concentration of 1 mm to improve digestion efficiency. Digestion products were separated on agarose gels (Sea Kem; FMC, Rockland, ME) and visualized by ethidium fluorescence. The DNA was blotted to uncharged nylon membranes (GeneScreen, New England Nuclear/DuPont, Boston/Wilmington, DE; Nytran, Schleicher & Schuell, Keene, NH) using the downward alkaline transfer protocol and Turboblotter apparatus (Schleicher & Schuell). Following transfer, the filters were neutralized, and the DNA was covalently linked to the filter by UV irradiation. Radiolabeled hybridization probes were generated by the random prime method (Feinberg and Vogelstein 1983). Hybridizations were done following the protocol of Church and Gilbert (1984). Filters were washed at 65° in 0.2× SSC, 0.1% SDS. Detection of the radiolabeled probes was done by autoradiography or phosphorimaging (Molecular Dynamics, Sunnyvale, CA). The following hybridization probes were generated from purified cloned inserts: 180-bp centromere repeat clone, pARR20-1 (Vongs *et al.* 1993); 5.8S-25S rRNA gene clone, pARR17 (Vongs *et al.* 1993); and a MHC9.7/9.8 subclone derived from m105 (Pruitt and Meyerowitz 1986). The *FWA* hybridization probe was generated by genomic amplification using the following oligonucleotide primers: 5′-CAGCGTCTACCAAATCTACACT-3′ and 5′-TAGTGTCTC GACAACGAACAAG-3′ (Soppe *et al.* 2000).

Quantification of 5-methylcytosine levels: Global cytosine methylation levels at CpG sites were estimated using a thin-layer chromatography assay as described by Kakutani *et al.* (1995). Briefly, total genomic DNA samples purified from either wild type or mutants (strain Columbia) were digested with the restriction enzyme *Taq*I (5′-T/CGA-3′). The terminal cytosines were radiolabeled *in vitro*, and the end-labeled DNA samples were enzymatically digested to mononucleotides. Methylated and unmethylated cytosine nucleotides were then separated by the thin-layer chromatography protocol described by Cedar *et al.* (1979) and quantified using phosphorimaging analysis (Molecular Dynamics).

DNA methyltransferase assays: Cytosine DNA methyltransferase activity was measured from nuclear extracts from axenic wild-type and *met1* mutant seedlings (10–14 days old, strain Columbia) as described in Kakutani *et al.* (1995). Briefly, proteins were solubilized from crude nuclear pellets with a buffer containing 0.2 m NaCl. Extracts were incubated with a hemi-methylated $(CpI)_n$ substrate and ^{3}H-labeled S-adenosylmethionine (methyl donor), and the amount of label transferred to DNA was measured after recovery on DE81 filters (Whatman).

DNA sequence determination: The coding sequence of the *MET1* gene was amplified by the polymerase chain reaction using KlenTaqI (DNA Polymerase Technology) polymerase and oligonucleotide primers distributed throughout the *MET1* genomic region. Nucleotide sequence was determined using BigDye terminator reagents and protocols (Perkin-Elmer, Norwalk, CT).

Molecular markers: Molecular cleaved amplified polymorphic sequence markers were developed to detect the *met1* alleles. The C → T *met1-1* mutation (corresponds to position 3898 in AB016872) was detected by genomic amplification (forward primer, 5′-CTCTTTAGTAGAAGTTGGCATG-3′; reverse primer, 5′-ATATGTATGTATAGATATTTTCTCC-3′), followed by *Hae*III digestion. The *met1-1* mutation destroys a *Hae*III site in the amplified fragment: wild type, 80 bp + 121

bp + 218 bp *vs. met1-1*, 121 bp + 298 bp. In a similar manner, the *met1-2* mutation (G → A at position 3301 in AB016872) can be detected by a creation of a *Bfa*I site. The *ddm1-2* allele was detected in segregating families by determining the nucleotide sequence of PCR-amplified genomic fragments flanking the mutation (forward primer, 5′-GCTGGAAGGGAAAGCT TAACAACCT-3′; reverse primer, 5′-ACACTGCCATCGATTC TGCAAACC-3′). The origin of the *FWA* allele in Columbia/Landsberg erecta segregating families was determined by examining the size of PCR-amplified products using the following primers: forward, 5′-CTGGTCAAGACTCTTATGGAC-3′ and reverse, 5′-ATTCCGCTTGTTCAATCCATG-3′, which detect an insertion of 94 bp within the seventh intron in the Landsberg erecta strain relative to the Columbia strain.

RESULTS

Isolation of *ddm2* mutants: We previously isolated three Arabidopsis DNA hypomethylation mutants using a Southern blot screen for mutant plants with centromeric repeats susceptible to digestion by the methylation-sensitive endonuclease, *Hpa*II (Vongs *et al.* 1993). We applied the screen to a different EMS-mutagenized population of Arabidopsis (strain Columbia) and identified four additional DNA hypomethylation mutants. Two of the mutations recovered in the new screen are recessive (see below) and allelic, but complemented hypomethylation mutations identified in the original screen that defined the *DDM1* (*decrease in DNA methylation 1*) locus (Vongs *et al.* 1993; Jeddeloh *et al.* 1999). The new mutations were originally designated *ddm2-1* and *ddm2-2*.

The *ddm2* mutations disrupt the *MET1* cytosine methyltransferase gene: An interstrain cross (*ddm2-1/ddm2-1* strain Columbia × wild-type strain Landsberg erecta) was used to generate a mapping population that segregated the *ddm2-1* mutation in the F_2 generation. F_2 individuals were scored for ribosomal RNA (rRNA) gene methylation using Southern blots, as described below. DNA samples from homozygous *ddm2-1* individuals were genotyped, using PCR-based markers that distinguish between parental strains. This analysis initially linked the *ddm2-1* mutation to the *DFR* and *LFY3* markers on the lower arm of chromosome 5 (http://www.arabidopsis.org). The *MET1* gene on the lower arm of chromosome 5 (Finnegan and Dennis 1993) encodes the primary Dnmt1-class maintenance cytosine methyltransferase in Arabidopsis. In our initial F_2 mapping population, there were no recombination events among 46 F_2 chromosomes between the *ddm2-1* mutation and the *MET1* gene (identified by an *Eco*RV restriction fragment length polymorphism).

We pursued the possibility that the *ddm2* mutations disrupt a cytosine DNA methyltransferase gene by assaying methyltransferase activity from *ddm2* mutants. Nuclear extracts from *ddm2-1* and *ddm2-2* homozygous mutant seedlings exhibited reduced cytosine DNA methyltransferase activity (data not shown; see materials and methods).

Our preliminary genetic mapping and biochemical data suggested that *MET1* was a likely candidate gene for disruption in the *ddm2* mutants. To identify changes in the *MET1* gene, we determined the nucleotide sequence of the *MET1* genomic region using templates generated by genomic amplification from the two *ddm2* mutants. The sequence of the entire *MET1* genomic region (~6 kb) was determined for both the *ddm2-1* and the *ddm2-2* alleles. In each case, a single G:C → A:T mutation was found that mapped to protein-coding exons. The *ddm2-1* allele is a missense mutation that replaces a proline with a serine residue in the catalytic domain of MET1 (P1300S, Figure 1A). The *ddm2-1* mutation does not alter any of the conserved motifs that define cytosine methyltransferases. The *ddm2-2* mutation replaces an invariant glycine residue with a serine in the signature methyltransferase motif I (G1101S, Figure 1B). The *ddm2-1* and *ddm2-2* alleles were renamed *met1-1* and *met1-2*, respectively.

DNA methylation phenotypes of *met1* mutants: Global cytosine methylation levels were estimated from the *met1* mutants using a thin-layer chromatography (TLC) approach to sample CpG methylation. *Taq*I-digested (T/CGA) genomic DNA from the two *met1* mutants, as well as *ddm1* and wild-type samples, were 5′-end labeled and then digested to mononucleotides. The methylation occupancy of the terminal cytosine was then measured after TLC separation as shown in Figure 2. *met1-1* homozygotes suffered a 70% reduction in cytosine methylation at TCGA sites, similar to that seen in *ddm1-2* homozygotes. A less severe reduction in DNA methylation to 50% wild-type levels was observed in *met1-2* homozygotes.

The genomic distribution of methylation in the mutants was surveyed by Southern blot analysis using the isoschizomers *Hpa*II and *Msp*I, which recognize 5′-CCGG-3′ sites. This site can be methylated at both cytosines in plants (Jeddeloh and Richards 1996). *Msp*I cannot digest ^{m}CCGG and *Hpa*II does so very inefficiently (http://rebase.neb.com/rebase/). However, *Msp*I can cleave this site when it is methylated at the internal cytosine (C^{m}CGG), while *Hpa*II cleavage is blocked.

In Figure 3A, we examined methylation at CCGG sites in the 180-bp centromeric repeat arrays. These repeat arrays are heavily methylated at C^{m}CGG in wild-type Columbia plants, as noted by the lack of *Hpa*II cleavage and the ability of *Msp*I to digest these genomic arrays. We included a control digest from a *ddm1* mutant, which contains DNA hypomethylated at both CpG and CpNpG sites. The more extensive cleavage of the centromere repeat arrays in the *ddm1-2 Hpa*II lane relative to the wild-type *Msp*I lane indicates that wild-type centromere arrays can be methylated at both cytosines in 5′-CCGG-3′. The *met1-1 Hpa*II profile resembles the wild-type *Msp*I sample (and *met1-1 Msp*I samples; data not shown), consistent with either a complete loss of ^{m}CpG or a partial loss of both ^{m}CpG and ^{m}CpNpG. *Hpa*II cleavage of the centromeric arrays was less extensive in *met1-2* mutants

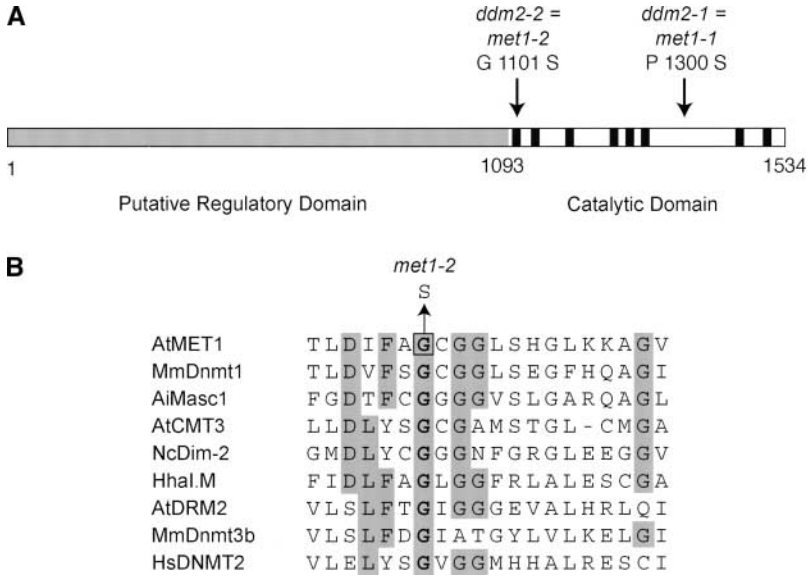

FIGURE 1.—MET1 protein structure and the *met1* missense mutations. (A) A schematic representation of the MET1 protein derived from DNA sequence. The large N-terminal domain (residues 1–1093) is likely to be involved in interactions with other proteins by analogy with other Dnmt1-class cytosine-DNA-methyltransferases. The black boxes that fall between residues 1094 and 1534 represent the regions encoding conserved motifs of the catalytic domain of the MET1 protein. The specific amino acid substitutions resulting from the *met1-1* and *met1-2* mutations are shown. (B) An alignment of motif I from a variety of different cytosine methyltransferases. The most conserved residues are shaded, and the invariant glycine residue is shown in boldface type. AiMasc1, Ascobolus Masc1 (AAC49849); AtCMT3, Arabidopsis CMT3 (AAK69756); AtDRM2, Arabidopsis DRM2 (AAF-66129); AtMET1, Arabidopsis MET1 (P34881); *Hha*I.M, Haemophilus *Hha*I methylase (P05102); HsDNMT2, human DNMT2 (O14717); MmDnmt1, mouse Dnmt1 (P13864); MmDnmt3b, mouse Dnmt3b (O88509); NcDim-2, Neurospora Dim-2 (AAK49954).

compared to *met1-1* mutants, indicating that *met1-2* causes a less severe reduction in cytosine methylation in this repetitive sequence.

The *met1* mutations also lead to a loss of cytosine methylation in other repetitive sequences. Figure 3B shows that the major rRNA gene repeats were strongly hypomethylated in both *met1-1* and *met1-2* homozygotes, indicated by the *Hpa*II digestion profiles. Surprisingly, the *met1-2* allele, which was identified as the weaker allele on the basis of the centromere repeat methylation phenotype, reduced rRNA gene repeat methylation to a level comparable to that caused by the *met1-1* allele. The *Hpa*II digestion profiles from the *met1* mutants show a similar or slightly greater cleavage relative to the wild-type *Msp*I profile, although the cleavage of the rRNA gene repeats at *Hpa*II sites was not as complete as that seen in *ddm1-2* homozygotes. These data suggest that the *met1* mutations lead to a loss of both ^{m}CpG and ^{m}CpNpG methylation, but caution is necessary when interpreting these data because *Msp*I cleavage can be blocked by CpG methylation in some sequence contexts (*e.g.*, GGC^{m}CGG is not cut by *Msp*I; BUSSLINGER *et al.* 1983).

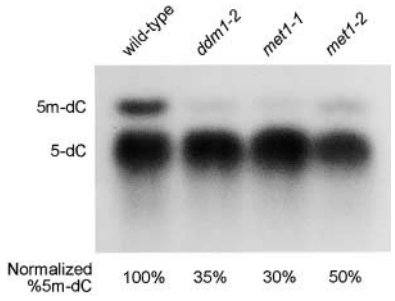

FIGURE 2.—Decrease in 5-methyl-cytosine in *met1* mutants. Loss of ^{m}CpG at 5′-TCGA-3′ sites was measured after thin-layer-chromatographic separation of *in vitro*-labeled terminal cytosines generated by *Taq*I restriction digestion of genomic DNA samples purified from wild-type, *ddm1-2*, *met1-1*, and *met1-2* homozygotes (Columbia strain). The mobility of 5-methyl-deoxycytidine monophosphate (5m-dC) and unmodified deoxycytidine monophosphate (5-dC) is indicated at the left.

met1 hypomethylation is not restricted to repetitive DNA sequences. Figure 3C shows a Southern filter hybridized with the MHC9.7/9.8 locus, an example of a methylated single-copy gene sequence in Arabidopsis (PRUITT and MEYEROWITZ 1986). In this case, the wild-type *Msp*I lane represents a complete digest at this genomic locus, while the wild-type *Hpa*II lane reflects the wild-type C^{m}CGG methylation pattern. The *met1-1* allele caused complete loss of MHC9.7/9.8 methylation, while the *met1-2* allele led to partial hypomethylation at this locus. The MHC9.7/9.8 sequence was also only partially hypomethylated in the *ddm1-2* mutant tested here. The partial hypomethylation profile seen in the *ddm1-2* *Hpa*II sample was consistent with our previous results, which demonstrated that the MHC9.7/9.8 locus becomes significantly hypomethlyated in *ddm1* mutants only after several generations of inbreeding (KAKUTANI *et al.* 1996).

Remethylation of DNA sequences hypomethylated in *met1-1* mutants: Genomic sequences stripped of cytosine methylation in *ddm1* mutants do not become remethylated when introduced into a wild-type background by genetic crosses (VONGS *et al.* 1993; KAKUTANI *et al.* 1999). We investigated whether sequences hypomethylated in *met1-1* mutants behave in a similar manner. Figure 4 shows that 180-bp centromere repeat arrays hypomethylated in *met1-1* mutants were partially but not fully remethylated in *MET1/met1-1* F_1 hybrids. Inheritance of the mutation and hypomethylated sequences through the fe-

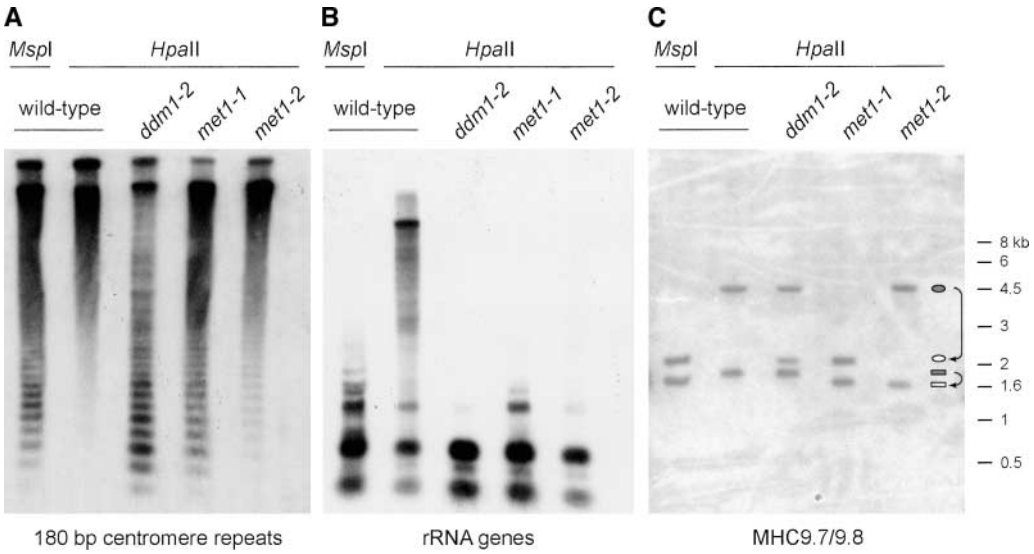

FIGURE 3.—The cytosine methylation phenotypes of *met1* mutants. Genomic DNA samples prepared from wild-type *MET1*, *ddm1-2*, *met1-1*, and *met1-2* homozygotes (Columbia strain) were digested with either *Msp*I or *Hpa*II and size fractionated by gel electrophoresis. After transfer to a nylon membrane, the DNA samples were hybridized sequentially with probes corresponding to the 180-bp centromere repeats (A), rRNA genes (B), or the MHC9.7/9.8 locus (C). The solid symbols in C represent restriction fragments resulting from methylated *Hpa*II sites; loss of DNA methylation at *Hpa*II sites leads to smaller fragments (open symbols). For the MHC9.7/9.8 locus, the different fragment shifts, 4.5 → 2.1 kb *vs.* 1.8 → 1.6 kb, represent loss of cytosine methylation at different *Hpa*II sites in the region. The *ddm1-2* mutation led to partial methylation at one *Hpa*II site, while the *met1-2* allele caused a complete loss of methylation at the other site. Molecular weight markers are shown to the right of C.

male or male lineage gave similar results. Thus, genomic sequences hypomethylated in *met1-1* mutants can be transmitted in a hypomethylated state through meiosis, similar to the situation for *ddm1*. However, *met1-1* hypomethylated centromere arrays were remethylated to some extent in the heterozygous F_1 hybrids, unlike the situation for *ddm1*.

Morphological phenotypes of *met1* mutants: *met1-2* homozygotes isolated in segregating populations exhibited normal morphology and development (data not shown). This result indicates that the 50% reduction in ^{m}CpG methylation conditioned by the *met1-2* allele is not severe enough to disrupt normal plant development. In contrast, the more dramatic reduction in ^{m}CpG methylation in *met1-1* mutants was associated with developmental abnormalities. Compared to wild-type plants, the most conspicuous phenotype of *met1-1* homozygotes was a delay in flowering time associated with the production of more rosette leaves and aerial (cauline) leaves prior to elongation of the flowering stem (Figure 5). Figure 6 shows the distribution of the flowering-time phenotypes relative to genotype in two families segregating the *met1-1* mutation: one in the Landsberg erecta strain background (introgressed through five backcrosses) and one in the Columbia strain background. Although absolute flowering time varied in the different strain backgrounds, the overall distribution of flowering time relative to *MET1* genotype was similar in Columbia and Landsberg erecta. The *met1-1* allele was recessive for phenotypic onset, but showed variable penetrance and expressivity in both backgrounds for the flowering-time phenotype.

To determine whether the late-flowering phenotype observed for *met1-1* homozygotes resulted from a delay during the vegetative phase of development, we measured the distribution of juvenile and adult rosette leaves in *met1-1* mutants. Juvenile rosette leaves are characterized by the absence of trichomes (hairs) on the abaxial (lower) leaf surface, while adult leaves possess abaxial trichomes (TELFER *et al.* 1997). The appearance of the first adult rosette leaf featuring an abaxial trichome was delayed in *met1-1* homozygotes compared to nonmutant sibling plants in both Columbia and Landsberg erecta strain backgrounds (Table 1). These data indicate that *met1-1* plants postpone the transition from juvenility to adulthood. In Landsberg erecta, the delay in this transition (~5 leaves) accounted for most of the late-flowering phenotype. However, the juvenile-adult transition delay (~2.4 leaves) in Columbia explains only a portion of the late-flowering phenotype in *met1-1* homozygotes. Several other morphological phenotypes accompanied the late-flowering phenotype in *met1-1* plants, including thick inflorescence stems, production of occasional aerial rosettes, and delayed senescence. It is not clear whether these additional *met1-1* phenotypes result from the flowering-time defect or are independent.

The late-flowering phenotype is caused by inherited variation unlinked to the *met1-1* mutation: A *met1-1* segregating family from an interstrain cross was generated to determine whether the late-flowering phenotype seen in the *met1-1* background mapped to the *MET1* locus. The *met1-1* Columbia homozygote with the most extreme late-flowering phenotype shown in Figure 6A was crossed to a wild-type Landsberg erecta individual, as well as a Columbia wild-type plant. *MET1/met1-1* hybrids resulting from the backcross to a wild-type Columbia plant had a flowering time intermediate between the

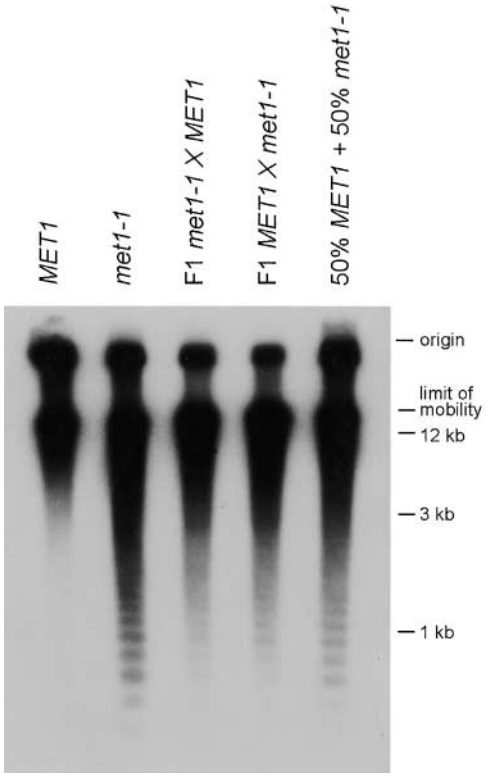

FIGURE 4.—Partial remethylation of centromere repeat arrays in *MET1/met1-1* F_1 hybrids. A Southern blot shows the 180-bp centromere repeat hybridization pattern after *Hpa*II digestion of genomic DNA samples prepared from wild-type *MET1* and *met1-1* homozygotes and F_1 hybrids from reciprocal crosses (Columbia strain). The control in the extreme right lane contains a 1:1 mixture of *MET1* and *met1-1 Hpa*II-digested genomic DNA that shows the hybridization pattern expected if no remethylation occurs in the F_1 hybrid. The mobility of molecular weight markers is shown at the right.

FIGURE 5.—Morphology of wild-type *MET1* and *met1-1* homozygotes (Columbia strain). Wild-type *MET1* (left) and *met1-1* (right) homozygous siblings were derived from a self-pollinated *MET1/met1-1* heterozygote. The *met1-1* homozygote exhibits a delay in flowering time that is accompanied by the production of additional rosette and cauline leaves before flowering stem elongation. The plants were genotyped as described in MATERIALS AND METHODS. Both plants are the same chronological age and were grown in parallel under the same environmental conditions.

parents (data not shown). In contrast, *MET1/met1-1* F_1 hybrids from the outcross to Landsberg erecta had an only slightly delayed flowering time (data not shown). Seeds from a self-pollinated F_1 *MET1/met1-1* Ler/Col individual were planted to generate a segregating F_2 family. As an indicator of flowering time, total leaf number (rosette plus cauline) at elongation of the flowering stem was measured for 67 F_2 individuals (Figure 7). Once the flowering stem elongated, leaf tissue was harvested and genomic DNA prepared to determine the *MET1* genotype as described in MATERIALS AND METHODS. The *met1-1* mutation segregated as expected (14 *MET1/MET1*:35 *MET1/met1-1*:18 *met1-1/met1-1*). The flowering times of the *met1-1* homozygotes in this population (see Figure 7) clustered toward the higher end of the distribution of phenotypes, consistent with our observations in pure genetic backgrounds (see Figure 6). However, almost one-half of the *MET1/MET1* and *MET1/met1-1* nonmutant plants were late flowering, suggesting that the late-flowering phenotype resulted from an alteration of at least one locus that segregates independently from *met1-1*.

Hypomethylation in *met1-1* mutants induces an *fwa* epigenetic allele: Loss of cytosine methylation in the upstream region of the *FWA* locus forms a stable epigenetic allele (epiallele) that results in the ectopic expression of a homeodomain protein, leading to late flowering (SOPPE *et al.* 2000). The methylation of the upstream region of the *FWA* gene was determined by Southern analysis using the methylation-sensitive *Cfo*I (G/CGC) restriction enzyme (Figure 8). *Cfo*I did not digest the *FWA* upstream region in the wild-type strains Columbia and Landsberg erecta, indicating that these restriction sites were fully methylated in wild-type *FWA* plants. The *FWA* locus was only partially demethylated in *ddm1-2* and *met1-2* mutants, which did not exhibit a delay in flowering time. However, *Cfo*I sites in the *FWA* upstream region were unmethylated in the *met1-1* mutants, as well as the *fwa* epigenetic mutant control. These

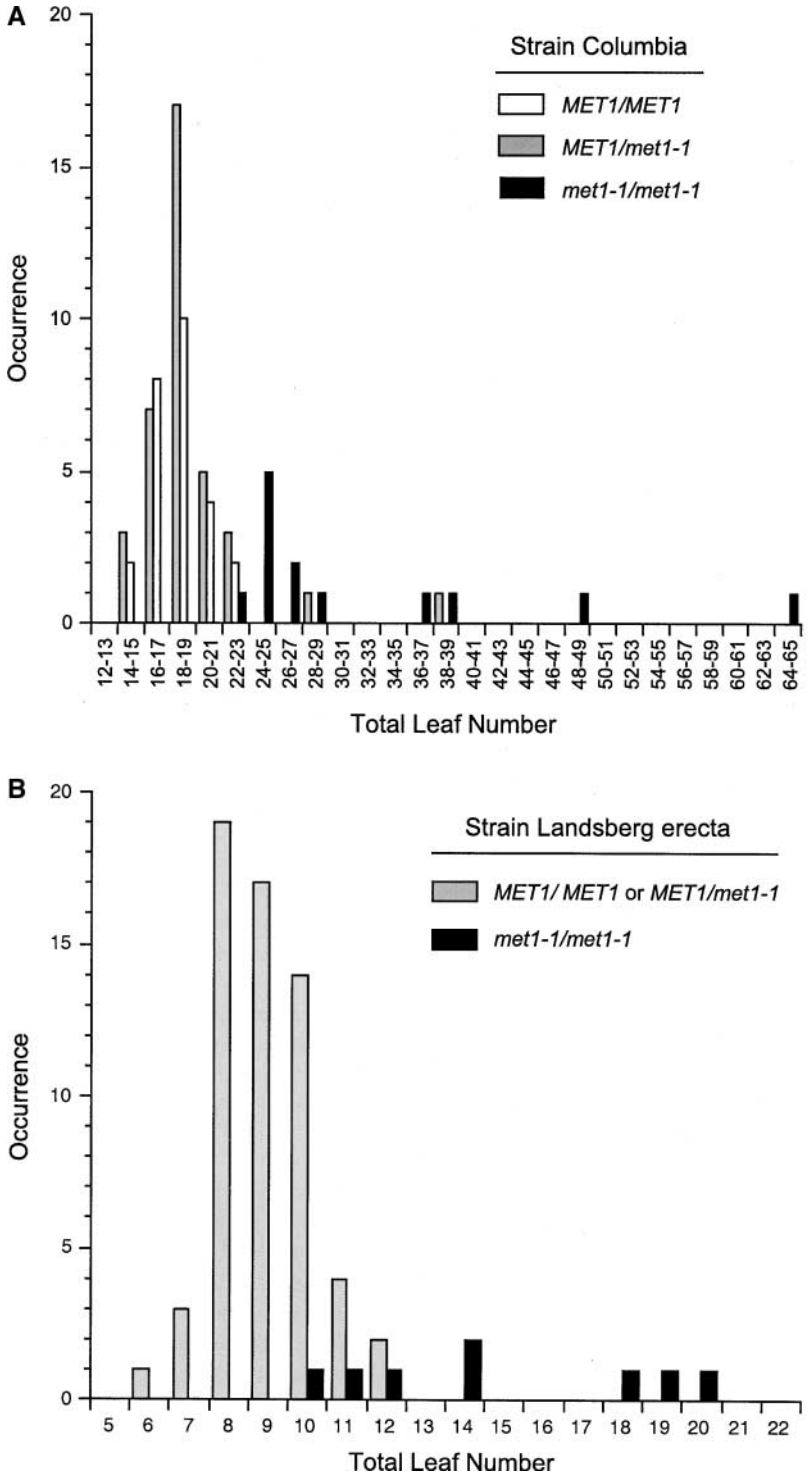

FIGURE 6.—The distribution of flowering-time phenotypes of F_2 populations segregating the *met1-1* allele in two different genetic backgrounds. Seeds from a self-pollinated F_1 *MET1/ met1-1* individual were used to generate an F_2 segregating family in strain Columbia (A) and in strain Landsberg erecta (B). Flowering time was determined for individuals by counting total leaf number (rosette plus cauline) at the initiation of flowering stem elongation. After flowering, the genotype at the *MET1* locus was determined for all individuals by either molecular genotyping (A; see MATERIALS AND METHODS) or Southern blot analysis (B).

observations suggest that the late-flowering phenotype observed for the *met1-1* mutants might result from hypomethylation at *FWA* and creation of an *fwa* epiallele.

Next, we examined selected individuals from the *met1-1* segregating F_2 family described in Figure 7, scoring both *FWA* methylation using Southern analysis and the parental origin of the *FWA* allele, to determine whether the formation and/or inheritance of hypomethylated *fwa* epialleles could account for the late-flowering phenotype. As expected, *FWA* hypomethylation was observed for all 10 *met1-1* homozygous F_2 plants examined (Figure 9, solid symbols). Several late-flowering *MET1/MET1* and *MET1/met1-1* F_2 individuals inherited and maintained either one or two *fwa* epialleles originating from the Col *met1-1* parent (Figure 9, asterisks). These results indicate that the *met1-1* mutation can create a stable, transmissible *fwa* epigenetic allele and that the late-flowering phenotype observed in *MET1/MET1* or

TABLE 1

Delay in the juvenile-to-adult rosette leaf transition in *met1-1* homozygotes

Strain	Genotype	First adult rosette leaf[a]
Columbia	*MET1/___*	6.8 ± 1.0 (n = 63)
Columbia	*met1-1/met1-1*	9.2 ± 1.6 (n = 13)
Landsberg erecta	*MET1/___*	4.1 ± 0.7 (n = 37)
Landsberg erecta	*met1-1/met1-1*	8.9 ± 1.6 (n = 9)

[a] Average ± standard deviation (n = number of individuals scored).

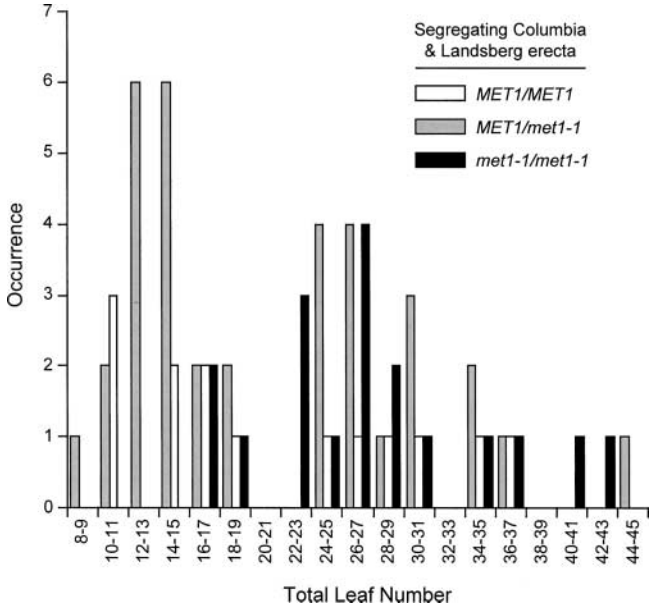

FIGURE 7.—The distribution of flowering-time phenotypes in a Columbia/Landsberg erecta F_2 population segregating the *met1-1* allele. A late-flowering Columbia *met1-1* mutant (shown in Figure 6A, 64 total leaves at flowering) was crossed as a female to a wild-type Landsberg erecta individual. Seeds from a self-pollinated F_1 individual were used to generate an F_2 segregating family. Flowering time was determined for 67 F_2 individuals by counting total leaf number (rosette plus cauline) at the initiation of flowering stem elongation. After flowering, the genotype of the *MET1* locus was determined for all 67 F_2 individuals as described in MATERIALS AND METHODS. The flowering times of control plants grown in parallel were Ler *MET1/MET1*, 11.8 ± 0.9 (SD) total leaves, n = 20; Col *met1-1/met1-1* late-flowering variant, 41.4 ± 2.8 (SD) total leaves, n = 20.

MET1/met1-1 F_2 plants can be explained, in part, by inheritance of *fwa* epigenetic alleles. However, we also noted exceptions to the simple Mendelian inheritance of Col *fwa* epialleles: two examples of *fwa* → *FWA de novo* methylation (up arrows, Figure 9) in *MET1/MET1* and *MET1/met1-1* individuals, as well as three examples of *FWA* → *fwa* hypomethylation (down arrows, Figure 9) in *MET1/met1-1* plants. Regardless, the segregation of additional genetic or epigenetic variation in this family must affect flowering time because the *fwa* epiallele cannot easily account for the extreme late-flowering phenotype of the Columbia *met1-1/met1-1* parent or the F_2 segregants with >30 total leaves. In addition, *fwa* epialleles are semidominant (SOPPE *et al.* 2000), but the late-flowering time behaved in a largely recessive manner in Col × Ler F_1 *MET1/met1-1* Ler/Col hybrids (see above).

Genetic interaction between *ddm1* and *met1* mutations: To determine the genetic interaction between *ddm1* and *met1* mutations, we generated a family in strain Columbia that segregated both mutations. Plants heterozygous for *ddm1-2* were crossed to plants heterozygous for *met1-1*. F_1 *trans*-heterozygotes were identified (see MATERIALS AND METHODS) and self-pollinated to generate *ddm1-2/ddm1-2 MET1/met1* and *DDM1/ddm1-2 met1-1/met1-1* mutants. These two types of F_2 plants were self-pollinated and *ddm1-2 met1-1* double-mutant homozygotes were recovered in numbers consistent with simple Mendelian segregation, arguing against defects in viability at any point in the life cycle. Figure 10 shows wild-type and single- and double-mutant plants with representative phenotypes. The *ddm1-2 met1-1* double mutants flowered late, as did the *met1-1* mutants, but the double mutant also displayed a darker color and more pronounced leaf curling, which were absent in both single mutants. The morphological phenotypes of double mutants were similar regardless of whether the double mutants were recovered from a family segregating *ddm1-2* or *met1-1*.

We subsequently monitored the distribution of genomic methylation at the single-copy sequence MHC9.7/9.8 and the repetitive 180-bp centromeric arrays and ribosomal RNA genes, using Southern blot analysis as described in Figure 3. In Figure 11A, we examined methylation at 5′-CCGG-3′ sites in the 180-bp centromeric repeat arrays. The centromere repeat arrays were cleaved more extensively in the *ddm1-2 MET1 Hpa*II lane relative to the *DDM1 met1-1 Hpa*II lane. *Hpa*II cleavage of the centromeric repeat arrays was more extensive in the *ddm1-2 met1-1* double mutants relative to the *met1-1* single mutant and resembled the profile seen for the *ddm1-2* single mutant.

The *Hpa*II digestion profile of the rRNA gene repeats indicates that *ddm1-2* and *met1-1* homozygotes were both capable of reducing methylation levels at these repeat sequences (Figure 11B). However, the cleavage of the rRNA gene repeats at *Hpa*II sites for the *met1-1* mutant was not as complete as that observed for *ddm1-2* homozygotes (Figure 11B). The *ddm1-2 met1-1* double-mutant *Hpa*II digestion profile at the rRNA gene repeats was very similar to that caused by the *ddm1-2* allele.

Finally, we monitored the extent to which the *ddm1-2 met1-1* double mutation combination affected methylation at the mHC9.7/9.8 single-copy locus. As described in Figure 3C, the *met1-1* allele caused a complete loss of MHC9.7/9.8 methylation, while the *ddm1-2* allele led

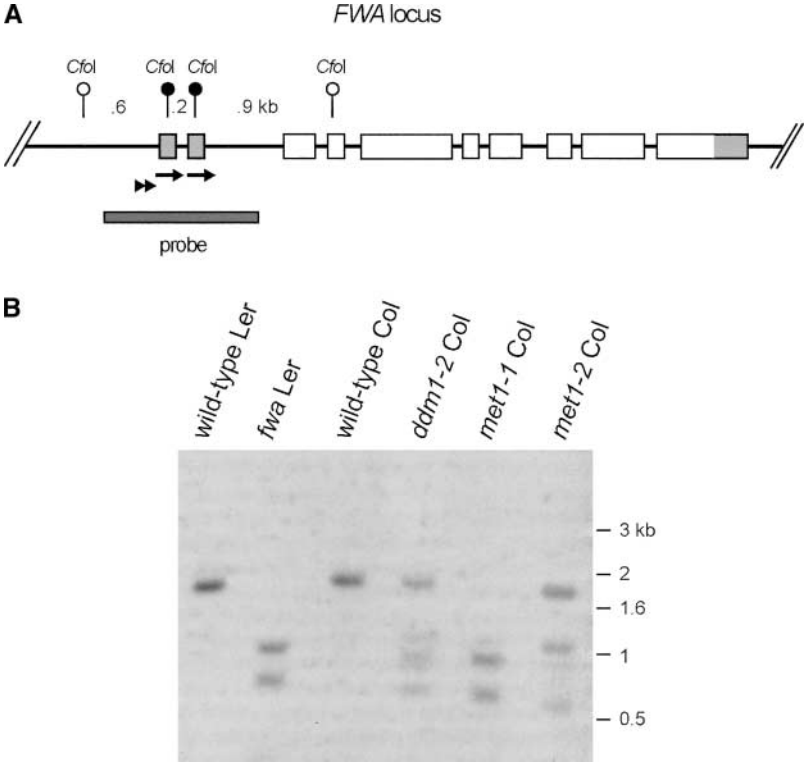

FIGURE 8.—Methylation of the *FWA* locus in *ddm1* and *met1* mutants. (A) Schematic map showing the structure of the *FWA* locus (open boxes, translated exons; shaded boxes, untranslated exons). Transcription proceeds from left to right. The arrows and arrowheads correspond to direct repeats in the upstream region. The position of the 1-kb hybridization probe within the *FWA* upstream region used for Southern analysis in B is shown, as well as the location of the relevant *Cfo*I restriction sites. The open circles indicate *Cfo*I sites that remain unmethylated and the solid circles represent *Cfo*I sites that are methylated in both wild-type Columbia and Landsberg erecta backgrounds. (B) The methylation of *Cfo*I restriction sites in the *FWA* upstream region was monitored by Southern analysis using the following genotypes: wild-type *FWA* (Columbia and Landsberg erecta), *fwa* (Landsberg erecta), *ddm1-2*, *met1-1*, and *met1-2*. The two internal *Cfo*I sites (solid circles in A) in the *FWA* upstream region remain methylated on the wild-type *FWA* allele in strain Columbia and Landsberg erecta plants. However, a complete loss of methylation at these sites was observed for *fwa* and *met1-1* mutant plants, whereas only a partial loss of methylation was seen at these sites for *ddm1-2* and *met1-2* mutant plants. Note that *ddm1-2* can give rise to stable, unmethylated *fwa* epialleles after repeated self-pollinations in *ddm1-2* homozygous lines.

to a partial loss of methylation at this locus. The *Hpa*II digestion profile of the MHC9.7/9.8 locus for the *ddm1-2 met1-1* double mutant was identical to that observed for the *met1-1* mutation (Figure 11C).

DISCUSSION

Here we describe the isolation and characterization of two EMS-induced alleles of the major Arabidopsis *Dnmt1*-class maintenance methyltransferase gene, *MET1*. The two *met1* missense mutations characterized map to the carboxy-terminal catalytic domain of the protein, although the stronger *met1-1* allele falls outside the eight methyltransferase signature motifs in the MET1 protein (FINNEGAN and DENNIS 1993). The cytosine methylation that remains in the *met1-1* homozygotes must originate from some residual MET1 function (if *met1-1* is a leaky allele) and/or from other cytosine methyltransferases encoded by the Arabidopsis genome.

The *met1-2* allele is notable for two reasons. First, although the global methylation assays indicate that *met1-2* is the weaker allele, this mutation alters an invariant glycine residue in the signature methyltransferase amino acid motif I (POSFAI *et al.* 1989), which is involved in binding the methyl donor S-adenosylmethionine and predicted to be essential for enzyme function (CHENG 1995). Second, the *met1-2* allele influences the genomic specificity of the methyltransferase. The *met1-2* allele has a very weak effect on centromere methylation relative to *met1-1*, despite the fact that the ribosomal RNA genes in *met1-2* homozygotes are hypomethylated to a degree comparable to that seen in *met1-1* mutants. The basis for this differential effect is not understood, but it indicates that the methyltransferase has some role in choosing targets.

The DNA methylation phenotype of the *met1* mutations differs from that of the previously described Arabidopsis cytosine hypomethylation mutations in the *DDM1* gene, which encodes a putative SWI2/SNF2 class chromatin remodeling protein (JEDDELOH *et al.* 1999). First, the *met1* mutations cause a reduction in cytosine methylation at both repetitive and single-copy sequences. In contrast, *ddm1* mutations lead to an immediate loss of methylation in the repetitive fraction of the genome but only a delayed and gradual hypomethylation of single-copy sequences (VONGS *et al.* 1993; KAKUTANI *et al.* 1996). Second, *ddm1* mutations reduce methylation of cytosines in all sequence contexts and appear to drive the highly repetitive sequences, like the centromere repeats and rRNA genes, to a nearly unmethylated state. In contrast, *met1* mutations lead to a dramatic loss of ^{m}CpG and a more modest reduction in ^{m}CpNpG in the repetitive fraction of the Arabidopsis genome. Early work suggested that MET1 acts primarily at CpG sites (FINNEGAN *et al.* 1996; KISHIMOTO *et al.* 2001), but more recent bisulfite sequencing results report a loss of both ^{m}CpNpG and methylation at asymmetric cytosines in *met1-1* mutants (BARTEE and BENDER 2001; LINDROTH *et al.* 2001), indicating that MET1 may have broad sequence specificity. Alternatively, non-CpG methylation may be reduced in *met1* mutants as a secondary conse-

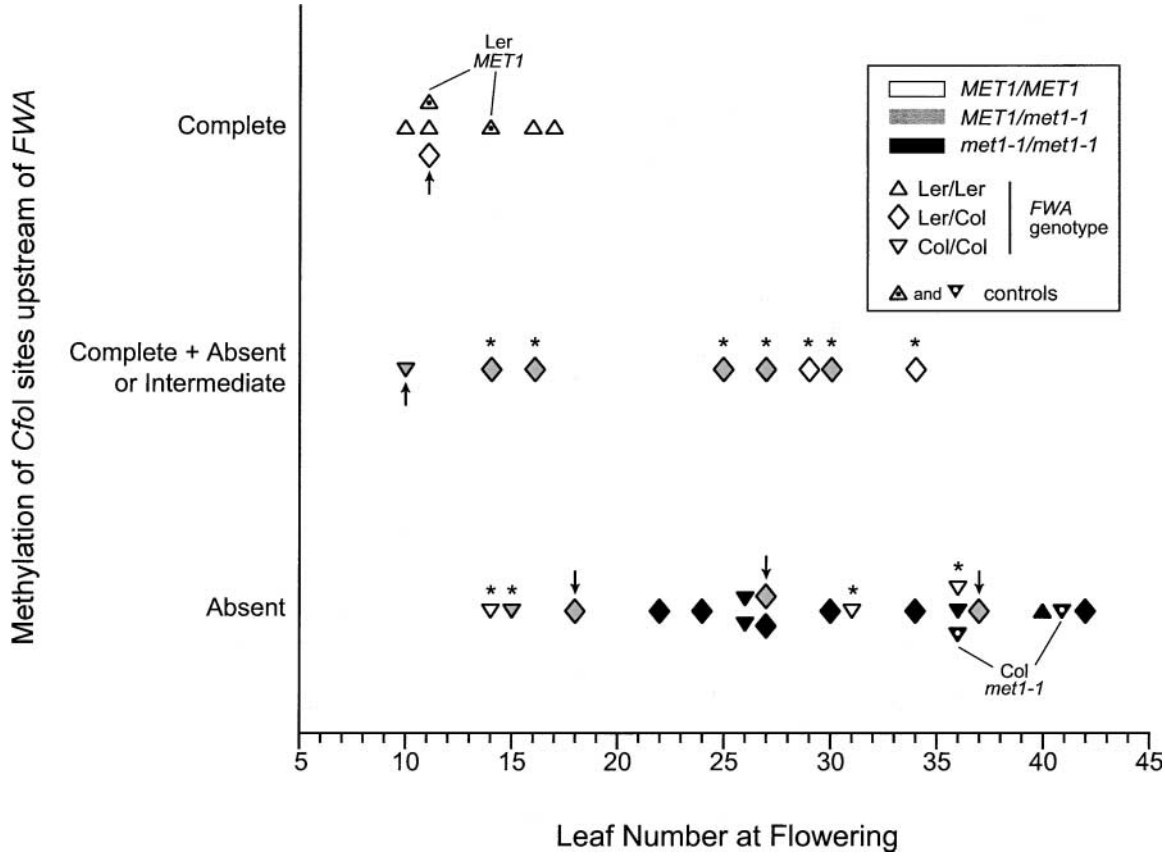

FIGURE 9.—Flowering time in a Columbia/Landsberg erecta F_2 population segregating *met1-1* and an *fwa* epiallele. The amount of hypomethylation in the upstream region of the *FWA* locus was plotted *vs.* total leaf number (rosette plus cauline) at flowering stem elongation for a subset of F_2 plants derived from the Columbia/Landsberg erecta F_2 population segregating the *met1-1* allele described in Figure 7. Cytosine methylation at the upstream *Cfo*I sites in *FWA* was determined by Southern hybridization analysis as described in Figure 8. The genotypes at the *MET1* and *FWA* loci were determined by PCR-based markers (see MATERIALS AND METHODS). Controls (symbols with dots) included progeny of the parents used in the original cross: a wild-type Landsberg erecta line (Ler *MET1*) and a Columbia late-flowering *met1-1* line (Col *met1-1*). Among the *MET1/MET1* and *MET1/met1-1* F_2 progeny, inheritance and maintenance of a hypomethylated *fwa* epiallele originating from the Col *met1-1* parent is common (asterisks). The arrows indicate either a loss of *FWA* methylation (down arrows) or *de novo* methylation of the *fwa* epiallele (up arrows) in *MET1/MET1* or *MET1/met1-1* F_2 progeny.

quence of a loss of ^{m}CpG (CAO and JACOBSEN 2002a). A third difference between *ddm1* and *met1* mutations concerns their ability to act at the haploid gametophyte stage. *ddm1* mutations do not hypomethylate the genome at the haploid stage (VONGS *et al.* 1993; KAKUTANI *et al.* 1999), but the hypomethylation of Ler *FWA* alleles in *MET1/met1-1* F_2 individuals (Figure 9, down arrows) suggests that *met1-1* may be acting at the haploid gametophytic stage. A fourth contrast between *ddm1*- and *met1*-induced cytosine hypomethylations is the fate of hypomethylated genomic sequences introduced into a wild-type background. The hypomethylated state induced by *ddm1* is stably inherited in crosses, and hypomethylated DNA originating from *ddm1* parents is maintained even in wild-type backgrounds (VONGS *et al.* 1993; KAKUTANI *et al.* 1999; SOPPE *et al.* 2002). Hypomethylation of genomic sequences caused by *met1-1* can also be transmitted and maintained in wild-type *MET1* individuals, but remethylation can also occur. This study provides two examples: (1) partial remethylation of centromere arrays in F_1 *MET1/met1-1* hybrids (Figure 4) and (2) remethylation of the *FWA* locus in *MET1/MET1* and *MET1/met1-1* F_2 individuals (Figure 9, up arrows). These findings are consistent with the results of RONEMUS *et al.* (1996), who demonstrated that remethylation of hypomethylated centromeric repeats can occur in individuals that have segregated away an antisense-*MET1* transgene.

The differences between the DNA methylation phenotypes of *met1* and *ddm1* mutations presumably reflect the different mechanisms of the corresponding wild-type proteins. While it is straightforward to suggest that MET1 has a direct role in cytosine methylation, the mechanism by which DDM1 acts is not understood. The difference in the efficiency of remethylation of *met1*- *vs.* *ddm1*-hypomethylated repetitive sequences may provide some clues. One possibility is that both *met1* and *ddm1* have a primary effect on DNA methylation. In *ddm1*

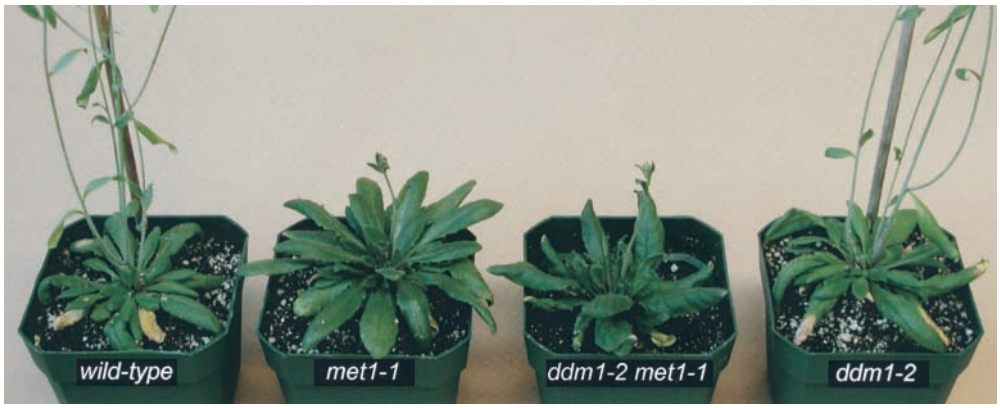

FIGURE 10.—Morphology of wild-type *MET1*, *met1-1*, *ddm1-2 met1-1*, and *ddm1-2* mutant plants. All plants were in the Columbia strain. The *met1-1* morphological phenotypes were similar to those seen in Figure 5. *ddm1-2* mutant plants closely resembled wild-type plants. The *ddm1-2 met1-1* double mutant displayed late flowering similar to *met1-1* mutants. In addition, the double mutants had a darker color than either single mutant, as well as a leaf-curling phenotype not seen in the single mutants. All plants were the same chronological age and were grown in parallel under the same environmental conditions.

mutants, most of the DNA methylation is removed from the repetitive sequences in all sequence contexts (*i.e.*, CpG, CpNpG, CpXpX), and remethylation may be difficult without preexisting cytosine methylation to mark the region for *de novo* methylation after inheritance through meiosis. In contrast, repetitive sequences inherited from *met1* mutants retain some methylation that may be sufficient to mark the region for at least partial remethylation upon introduction into a wild-type background. Support for the latter hypothesis comes from our unpublished results with inbred *met1-1* homozygotes with more severe centromere hypomethylation phenotypes. In these cases, centromere hypomethylation is more prominent in the F_1 hybrids resulting from outcrosses to wild-type plants (see also SOPPE *et al.* 2002). An alternative model posits that DDM1 is important for establishing a non-5mC epigenetic mark that guides the *de novo* methylation machinery. Recent reports demonstrate that histone H3 methyl-lysine 9 provides a chromatin mark important for DNA methylation (TAMARU and SELKER 2001; JACKSON *et al.* 2002) and that the *ddm1* mutation leads to depletion of this histone methylation mark in heterochromatin (GENDREL *et al.* 2002; JOHNSON *et al.* 2002). Under this scenario, unmethylated genomic sequences fail to become remethylated because they have lost the histone H3 methyl-lysine 9 mark. However, the precipitous loss of DNA methylation in *ddm1* homozygous progeny of self-pollinated fully methylated *DDM1/ddm1* parents suggests that DDM1 is necessary for maintenance of cytosine methylation (VONGS *et al.* 1993; JEDDELOH *et al.* 1998), not for specification of *de novo* methylation. In addition, mechanisms for depleting histone H3 lysine 9 methylation as a consequence of a loss of cytosine methylation are now coming into focus (RICHARDS 2002; FUKS *et al.* 2003). Indeed, it may be impossible to define a simple linear cause-and-effect signaling pathway; rather, a network of self-reinforcement among the 5mC, histone H3 methyl-lysine 9, and other nongenetic marks may operate at the core of epigenetic signaling (RICHARDS and ELGIN 2002).

We sought further insight into the mechanistic relationship between MET1 and DDM1 by examination of *ddm1 met1* double mutants. Our *ddm1 met1* double-mutant analysis suggested a complex, nonadditive interaction between these mutations in terms of gross morphology and developmental characters. With regard to the DNA methylation phenotype, the *ddm1-2 met1-1* double mutants have a repetitive DNA methylation pattern that resembles the more severe hypomethylation caused by the *ddm1-2* mutation (see Figure 11, A and B), but methylation of the single-copy MHC9.7/9.8 locus in *ddm1-2 met1-1* double mutants is equivalent to that seen in the *met1-1* parent rather than that in *ddm1-2* mutants (see Figure 11C). On the basis of these results we formally can define *ddm1-2* as epistatic to *met1-1* for repetitive DNA methylation while specifying *met1-1* as epistatic to *ddm1-2* for single-copy sequence methylation. However, if we consider the DNA methylation phenotype as a composite, the effects of the *ddm1-2* and *met1-1* mutations are additive, consistent with DDM1 and MET1 acting independently to effect cytosine methylation. We note that BARTEE and BENDER (2001) found *ddm1-2* to be epistatic to *met1-1* for the differential hypomethylation effects seen at the *PAI* loci. It is necessary to be cautious when interpreting these genetic interactions, particularly in light of the fact that there are many Dnmt1-class methyltransferase genes in Arabidopsis and the *met1-1* mutation may not be a null allele.

The most conspicuous phenotype of *met1-1* homozygotes is a delayed shift from the vegetative to the reproductive phase of development. This phenotype has at least three interrelated components: (1) a prolonged juvenile phase of vegetative development, (2) a tempo-

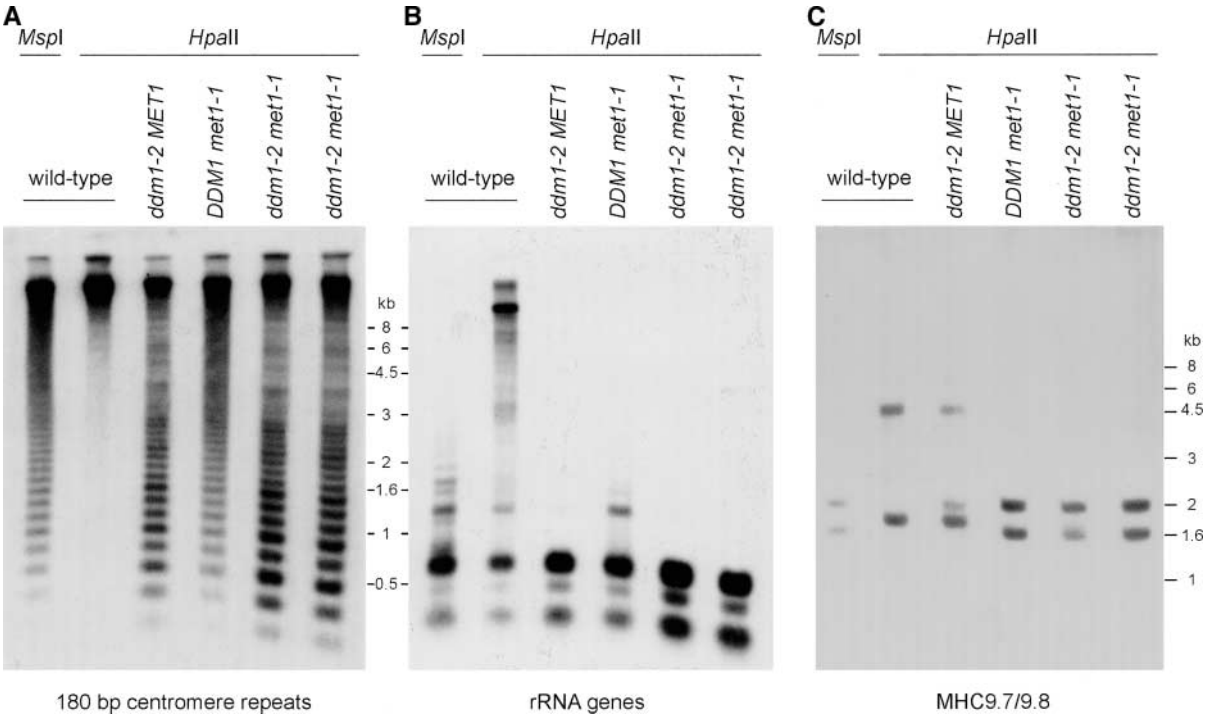

FIGURE 11.—The cytosine methylation phenotypes of *ddm1-2 met1-1* double mutants. Genomic DNA samples prepared from wild-type, *ddm1-2 MET1*, *DDM1 met1-1,* and *ddm1-2 met1-1* homozygotes (Columbia strain) were digested with either *Msp*I or *Hpa*II and size fractionated by gel electrophoresis. After transfer to a nylon membrane, the DNA samples were hybridized sequentially with probes corresponding to the 180-bp centromere repeats (A) and the rRNA genes (B). An independent gel and filter were prepared for hybridization with the MHC9.7/9.8 locus (C).

ral delay in the initiation of the flowering stem, and (3) an increase in the number of rosette and aerial (cauline) leaves produced before initiation of the flowering stem. RONEMUS *et al.* (1996) also reported that late flowering was a common phenotype in antisense-*MET1* plants (strain Columbia). In our experiments, the penetrance and expressivity of the *met1* late-flowering phenotype were variable (see Figure 6). The late-flowering defect was caused by newly generated variation that segregated independently of the *met1-1* mutation in an F_2 mapping population (see Figure 7). The complexity of the distribution in phenotypes in the mapping population suggests that several loci may be involved. Hypomethylation of the *FWA* locus was identified as one specific target of the *met1-1* mutation, and the formation of stable epigenetic *fwa* epialleles accounted for some of the late-flowering phenotype (see Figure 9). At present, the identities of other alterations contributing to *met1-1*-induced late flowering are unknown. In addition, some of the variability in the phenotypes seen in Figures 7 and 9 may be caused by segregation of modifier loci present in the polymorphic Col and Ler parents used in the mapping cross (UNGERER *et al.* 2002).

Variable phenotypic severity/onset and formation of stable variation unlinked to the *met1-1* mutation closely parallel the mutator-like phenomenon displayed by inbred *ddm1* mutants (KAKUTANI *et al.* 1996; KAKUTANI 1997). Accumulation of both random transposon insertions (MIURA *et al.* 2001; SINGER *et al.* 2001) and more directed, stable epigenetic alleles at a limited number of genomic sites (KAKUTANI 1997; SOPPE *et al.* 2000; STOKES *et al.* 2002) is responsible for the array of phenotypes that arise in *ddm1* lines. We are currently inbreeding the *met1-1* lines to determine if a similar range of stochastic phenotypes develops. An independent group has demonstrated that hypermethylated *sup* epialleles arise sporadically, but at a high frequency, in inbred *met1-1* lines (JACOBSEN *et al.* 2000).

The data presented here demonstrate that the cytosine methyltransferase MET1 is essential for the maintenance of global cytosine methylation in Arabidopsis, despite the presence of several other methyltransferase genes, including *Dnmt1*-class genes. Further, the consequence of loss of *MET1* function closely parallels the (epi)mutator phenomenon seen in *ddm1* mutants. Given that both DDM1 and MET1 are required for normal cytosine methylation, the simplest explanation is that this fundamental covalent DNA modification is necessary for the integrity and stability of genomic information (CHEN *et al.* 1998).

We thank Amy Ripperger, Courtney Keane, Anna Korn, Greg Jacobsen, Jennifer Agnew, Anne Swart, Hankuil Yi, and Joe Jarvis for technical assistance on various aspects of the project. We express special thanks to Brian Drew, Rebecca Dunlap, and Michele Walchko for conducting the initial mutant screen that identified the *met1* mutants and to Tetsuji Kakutani for his experimental suggestions regarding

fwa. We thank the Arabidopsis Biological Resource Center at The Ohio State University for providing the *fwa* reference allele and various wild-type strains. This work was supported by grants from the National Science Foundation to E.J.R (MCB-9306266, MCB-9604972, and MCB-9985348) and by predoctoral fellowships from the Monsanto Company to J.A.J. and T.L.S. D.E.R was supported in part by a WU/HHMI Summer Undergraduate Research Fellowship funded by an Undergraduate Biological Sciences Education Program grant from the Howard Hughes Medical Institute to Washington University.

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Communicating editor: S. Henikoff