Arabidopsis MET1 Cytosine Methyltransferase Mutants


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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 fua 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 (Hol-liday 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 (Asco-bolus) 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
enzymes within the plant nucleus is beginning to be understood. Antisense suppression of the Arabidopsis Drmt1-class gene MET1 caused a reduction of global cytosine methylation levels, particularly at CpG sites (FinneGAN et al. 1996; RonEamus 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 60% Redi-Earth (Scotts):40% vermiculite mixture. M2 plants were grown under standard greenhouse conditions with supplemental light during the summer of 1994. DNA hypomethylation mutants were identified by loss of HpaII 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 proteins were solubilized from crude nuclear pellets with a

ddm1-2 met1-1

met1-2

ddm1-2 MET1/DDM1 met1-1 trans

ual to generate a

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°C 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 (Prutt and Meyerowitz 1986). The FWA hybridization probe was generated by genomic amplification using the following oligonucleotide primers: 5′-CAGGCGTCTACAAAATCTCAGC-3′ and 5′-TAGTGCTTCAGAAAACGACAG-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 Kaktan et al. (1995). Briefly, total genomic DNA samples purified from either wild type or mutants (strain Columbia) were digested with the restriction enzyme TaqI (5′-T/CAG-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 Kaktan 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), substrate and 3H-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′-CTCTTTAGTGAAGTGGCATG-3′; reverse primer, 5′-ATATGTATGTATAGATATTTTCTCC-3′), followed by HaeIII digestion. The met-1 mutation destroys a HaeIII site in the amplified fragment: wild type, 80 bp + 121

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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 BfaI 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'-GCTGGAAGGGAAGGCTTAACAAGCT-3'; reverse primer, 5'-ACACTGCCATCGATTCGTAACAGC-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'-CTGCTGCAAGACTCTATGGAC-3' and reverse, 5'-ATTCCGCTTGTCAATCCATG-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, HpaII (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 mutant, which contains DNA hypomethylated at both CpG and CpNpG (identified by an EcoRV 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 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. TaqI-digested (T/C) 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 homoygotes suffered a 70% reduction in cytosine methylation at TCGA sites, similar to that seen in ddm1-2 homoygotes. A less severe reduction in DNA methylation to 50% wild-type levels was observed in met1-2 homoygotes.

The genomic distribution of methylation in the mutants was surveyed by Southern blot analysis using the isoschizomers HpaII and MspI, which recognize 5'-CCGG-3' sites. This site can be methylated at both cytosines in plants (Jeddeloh and Richards 1996). MspI cannot digest 5'-CCGG and HpaII does so very inefficiently (http://rebase.neb.com/rebase/). However, MspI can cleave this site when it is methylated at the internal cytosine (CmCCG), while HpaII 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 CmCCG in wild-type Columbia plants, as noted by the lack of HpaII cleavage and the ability of MspI 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 HpaII lane relative to the wild-type MspI lane indicates that wild-type centromere arrays can be methylated at both cytosines in 5'-CCGG-3'. The met1-1 HpaII profile resembles the wild-type MspI sample (and met1-1 MspI samples; data not shown), consistent with either a complete loss of 5'-CpG or a partial loss of both 5'-CpG and 5'-CpNpG. HpaII 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. AtMasc1, Ascobolus Masc1 (AAC49849); AtCMT3, Arabidopsis CMT3 (AAK69756); AtDRM2, Arabidopsis DRM2 (AAF6129); AtMET1, Arabidopsis MET1 (P34881); HhaI.M, Haemophilus HhaI methylase (P05102); HsDNMT2, human DNMT2 (O14717); MmDnmt1, mouse Dnmt1 (P13864); MmDnmt3b, mouse Dnmt3b (O88509); NcDim-2, Neurospora Dim-2 (AAK49954).

Figure 2.—Decrease in 5-methyl-cytosine in met1 mutants. Loss of \(^{32}CpG\) at 5'-TCGA-3' sites was measured after thin-layer-chromatographic separation of \(^{32}P\)-labeled terminal cytosines generated by TaqI 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.
DNA Methyltransferase Mutants

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 MspI or HpaII 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 HpaII sites; loss of DNA methylation at HpaII 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 HpaII sites in the region. The ddm1-2 mutation led to partial methylation at one HpaII 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 F1 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 5mCpG methylation conditioned by the met1-2 allele is not severe enough to disrupt normal plant development. In contrast, the more dramatic reduction in 5mCpG 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 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 F1 hybrids, unlike the situation for ddm1. 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

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Figure 4.—Partial remethylation of centromere repeat arrays in MET1/met1-1 F1 hybrids. A Southern blot shows the 180-bp centromere repeat hybridization pattern after HpaII digestion of genomic DNA samples prepared from wild-type MET1 and met1-1 homozygotes and F1 hybrids from reciprocal crosses (Columbia strain). The control in the extreme right lane contains a 1:1 mixture of MET1 and met1-1 HpaII-digested genomic DNA that shows the hybridization pattern expected if no remethylation occurs in the F1 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.

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 CfoI (G/CGC) restriction enzyme (Figure 8). CfoI 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, CfoI sites in the FWA upstream region were unmethylated in the met1-1 mutants, as well as the fwa epigenetic mutant control. These
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 F2 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 F2 plants examined (Figure 9, solid symbols). Several late-flowering MET1/MET1 and MET1/met1-1 F2 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...
MET1/met1-1 F2 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 F2 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 F1 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. F1 trans-heterozygotes were identified (see materials and methods) and self-pollinated to generate ddm1-2/ddm1-2 MET1/met1-1 and DDM1/ddm1-2 met1-1/met1-1 mutants. These two types of F2 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 HpaII lane relative to the DDM1 met1-1 HpaII lane. HpaII 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 HpaII 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 HpaII 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 HpaII 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

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**TABLE 1**

| Strain                  | Genotype | First adult rosette leaf
d | n  |
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Columbia</td>
<td>MET1/___</td>
<td>6.8 ± 1.0 (n = 63)</td>
</tr>
<tr>
<td>Columbia</td>
<td>met1-1/met1-1</td>
<td>9.2 ± 1.6 (n = 13)</td>
</tr>
<tr>
<td>Landsberg erecta</td>
<td>MET1/___</td>
<td>4.1 ± 0.7 (n = 37)</td>
</tr>
<tr>
<td>Landsberg erecta</td>
<td>met1-1/met1-1</td>
<td>8.9 ± 1.6 (n = 9)</td>
</tr>
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*Average ± standard deviation (n = number of individuals scored).
to a partial loss of methylation at this locus. The HpaII 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 11G).

**DISCUSSION**

Here we describe the isolation and characterization of two EMS-induced alleles of the major Arabidopsis Ddm1-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 (Posee 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 CpG and a more modest reduction in 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 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-

![DNA Methyltransferase Mutants](1117DNA Methyltransferase Mutants)
Figure 9.—Flowering time in a Columbia/Landsberg erecta F2 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 F2 plants derived from the Columbia/Landsberg erecta F2 population segregating the met1-1 allele described in Figure 7. Cytosine methylation at the upstream CfoI 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 F2 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 F2 progeny.

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 met1-1 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
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 homozygous with more severe centromere hypomethylation phenotypes. In these cases, centromere hypomethylation is more prominent in the F1 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 (Vong 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 MspI or HpaII 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).

...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 mutation in an F2 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 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-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).

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