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Characterization of an *Arabidopsis thaliana* DNA hypomethylation mutant

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ABSTRACT

We have recently isolated two *Arabidopsis thaliana* DNA hypomethylation mutations, identifying the *DDM1* locus, that cause a 70% reduction in genomic 5-methylcytosine levels [1]. Here we describe further phenotypic and biochemical characterization of the *ddm1* mutants. *ddm1/ddm1* homozygotes exhibited altered leaf shape, increased cauline leaf number, and a delay in the onset of flowering when compared to non-mutant siblings in a segregating population. Our biochemical characterization investigated two possible mechanisms for DNA hypomethylation. In order to see if *ddm1* mutations affect DNA methyltransferase function, we compared DNA methyltransferase activities in extracts from wild-type and *ddm1* mutant tissues. The *ddm1* mutant extracts had as much DNA methyltransferase activity as that of the wild-type for both the CpI and CpNpG substrates suggesting that the *DDM1* locus does not encode a DNA methyltransferase. Moreover, the *ddm1* mutations did not affect the intracellular level of S-adenosylmethionine, the methyl group donor for DNA methylation. The possibility that the *DDM1* gene product functions as a modifier of DNA methylation is discussed.

INTRODUCTION

A large body of evidence indicates that cytosine methylation in eukaryotes plays a role in modulating gene expression [2, 3], genome organization [4], and chromosome behavior [5]. We are using the flowering plant *Arabidopsis thaliana* to pursue a genetic approach to the study of DNA methylation. We have recently isolated two *A.thaliana* DNA hypomethylation mutations that identify a locus, *DDM1*, important for cytosine methylation [1]. Genomic 5-methylcytosine content is reduced to approximately 30% of wild-type levels in *ddm1* homozygotes. The *ddm1* mutations cause hypomethylation of both repetitive and single-copy sequences, and affect CpG as well as CpNpG sites. The hypomethylation caused by the *ddm1* lesions exhibits site-specificity; some single-copy DNA sequences remain methylated in *ddm1* mutants. Despite the large reduction in cytosine methylation, our initial qualitative phenotypic analysis of the *ddm1* homozygotes did not reveal dramatic morphological abnormalities. Here, we present a more rigorous quantitative examination of the *ddm1* morphological phenotype.

Eukaryotic DNA methylation mutants have been reported in two other model systems. Li *et al.* described a mouse DNA hypomethylation mutation, created by disruption of a cytosine methyltransferase gene, that leads to an approximately 70% reduction in genomic methylation levels [6]. Murine embryonic stem cell lines homozygous for the engineered mutation are viable, yet animals homozygous for the mutation develop abnormally and die as early embryos. Recently, a number of *Neurospora* mutations (*dim*) were isolated that cause a decrease in genomic 5-methylcytosine content [5]. These mutations, which define three loci important for genomic cytosine methylation, do not express dramatic morphological phenotypes, despite the fact that at least one of the mutations leads to the absence of detectable 5-methylcytosine. The biochemical basis of the DNA hypomethylation caused by the *Neurospora dim* mutations has not been reported.

In this report, we investigate possible mechanisms by which the *A.thaliana ddm1* mutations affect DNA methylation. One possibility is that the *ddm1* mutations disrupt a DNA methyltransferase gene. DNA methyltransferase mutations that lead to DNA hypomethylation have been isolated in *E.coli* [7, 8] and engineered in mice [6]. Alternatively, the *A.thaliana ddm1* mutations may cause DNA hypomethylation by reducing the intracellular concentration of S-adenosylmethionine (Ado-met), the methyl group donor for DNA methylation. Recent work using *Neurospora* conditional mutants deficient in Ado-met metabolism demonstrate that Ado-met starvation can cause DNA hypomethylation [5].

A third possibility is that the *DDM1* gene product regulates DNA methylation. The molecular mechanisms establishing DNA methylation patterns of eukaryotic genomes are not well understood. Methylated cytosines are not randomly distributed in the genome, although eukaryotic DNA methyltransferases do not exhibit much sequence-specificity *in vitro* beyond the boundaries of the symmetrical sites methylated (e.g. CpG and CpNpG in plants) [9, 10]. Experiments in mice indicate that DNA methyltransferase activity is regulated. For example, tissues with different methylation patterns appear to have identical forms of DNA methyltransferases [11]. A dramatic intracellular redistribution of DNA methyltransferase, between the cytoplasm and nucleus, occurs during early mouse development [12]. Surprisingly,

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increased nuclear localization of the DNA methyltransferase during preimplantation mouse development does not correlate with an increase in genomic methylation, further suggesting the action of regulatory factors. In addition, a number of modifier loci that affect DNA methylation of transgenes have been documented in mouse [13–15]. These considerations support the existence of factors that modify DNA methyltransferase activity.

To begin to distinguish among these possibilities, we examined DNA methyltransferase activity and Ado-met levels in *ddm1* mutant and wild-type backgrounds. Our results suggest that the *DDM1* gene product is neither a DNA methyltransferase nor a factor affecting Ado-met pool size. The possibility that *DDM1* product functions as a modifier of DNA methylation will be discussed.

MATERIALS AND METHODS

Plant and cell culture

Isolation and initial characterization of *ddm1* mutants were reported by Vongs *et al.* [1]. The *ddm1-2/ddm1-2* mutant line used was backcrossed to the parent strain, Columbia, for six generations to remove unlinked mutations. Pea seeds (*Pisum sativum* var. Extra Early Alaska) were purchased from Greenseed, Kimberly, Idaho. Tissue for enzyme assays was derived from plants grown in a glasshouse at approximately 25°C. Callus cultures were generated from sterilized seeds, as described by Doelling and Pikaard [16], and were maintained by transferring to fresh solid media approximately once every three weeks.

Phenotypic analysis

Seeds from self-pollination of a *ddm1-2/+* heterozygote (backcrossed six times to parental strain, Columbia) were used to generate two independent segregating populations. Plants were grown as individuals in separate three inch pots (100 pots each) in a controlled environmental chamber (21°C, 75% relative humidity, mixed fluorescent/incandescent illumination for 14 h per day). Plants were monitored daily to check for germination and flowering onset. Leaf numbers (cauline and rosette) were counted at flowering onset. The sixth rosette leaf to emerge was removed at flowering onset and pressed to a paper substrate in preparation for leaf shape analysis. Leaf shape (area/width^2) was measured using a video imaging system and MorphoSys software (Meacham, C.A. and Duncan, T., University of California; Version 1.26).

Preparation and analysis of plant DNA

A.thaliana DNA was prepared by a modification of the urea lysis method (Karen Cone, personal communication). Southern analysis of the genomic DNA was performed as described by Ausubel *et al.* [17] using the high SDS hybridization buffers of Church and Gilbert [18]. Radiolabeled probe of the insert from the *A.thaliana* 180 bp centromere repeat clone, pARR20-1 [1], was generated by the random priming method [17].

Nuclear preparation and solubilization of DNA methyltransferase

We modified the protocol of Yesufu *et al.* [19] for preparation and storage of plant nuclei. 1–8 g of calli were used for *A.thaliana* nuclei preparation. For preparation of pea nuclei, 1.5 cm from the apical tip of six days old pea shoots were used. Tissue was homogenized in Buffer M (10% glycerol, 50 mM MOPS/NaOH pH 7.2, 1 mM EDTA, 0.01% NaN_3, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride), and a crude nuclear pellet was recovered by centrifugation after filtration through Miracloth (Cal Biochem). The pellet was resuspended in Buffer M containing 0.5% Triton X-100 to lyse chloroplasts, and the nuclei were collected by centrifugation. This step was repeated (once or twice) until the nuclear pellet no longer was green. The pellet was then washed by resuspension in Buffer M, followed by centrifugation. The final pellet was resuspended in Buffer M containing 50% glycerol and the nuclear suspension stored at –20°C. Solubilization of the DNA methyltransferase activity was performed in Buffer M containing 0.2 M NaCl as described by Houlston *et al.* [20].

Synthesis of substrate DNA for methyltransferase reactions

Hemimethylated DNA substrates were made by the polymerization method of Giordano *et al.* [21]. Hemimethylated poly [d(CI)] was synthesized *in vitro* using the Klenow fragment, poly [d(CI)] template DNA and a nucleotide mixture of 0.25 mM 5-methyl-dCTP, 0.75 mM dCTP, and 1 mM dITP. Hemimethylated poly [d(CNG)] was made using chemically synthesized $[d(CAG)]_7$ and $[d(CTG)]_7$ as template (3.6 µM each) and a nucleotide mixture of 1 mM dGTP, 0.5 mM dATP and dTTP, 0.75 mM dCTP and 0.25 mM 5-methyl-dCTP. Control substrates were made in parallel with nucleotide mixtures omitting 5-methyl-dCTP. During these reactions, poly [d(CI)] elongated from less than 70 bases to more than 500 bases, and poly [d(CNG)] elongated from 21 bases to about 80 bases (assayed by alkaline gel electrophoresis). Efficiency of the elongation reactions were comparable between the hemimethylated and unmethylated DNA.

DNA methyltransferase assay

DNA methyltransferase activity was measured essentially as described by Yesufu *et al.* [19]. The assay mixture (65 µl) contained 10 µl of plant cell-free extracts, 50 µl of reaction mix (40 mM Tris–HCl 7.2, 1 mM EDTA, 1 mM dithiothreitol and 10% glycerol), 4 µl of S-adenosyl-L-[methyl ^{3}H]-methionine (DuPont, 0.5 µCi/µl) and 1 µl of substrate DNA (5 µg/µl). Hemimethylated poly [d(CI)] was used as substrate DNA except where noted. Reaction mixtures were incubated at 25°C and were stopped by two extractions with phenol/chloroform. The aqueous phase was then adjusted to 0.1 M NaOH and incubated at 50°C for 2 h to degrade RNA. After neutralization with HCl, the radioactivity binding to DE 81 paper (Whatman) was measured by scintillation counting. Control reactions were performed without substrate DNA to determine background levels. Protein concentrations were estimated by the method of Bradford using bovine serum albumin standards [17].

Thin-layer chromatography and identification of methyl group acceptor

After phenol/chloroform extraction and NaOH treatment, DNA methyltransferase reaction products were concentrated by ethanol precipitation and resuspended in TE buffer (10 mM

Tris–HCl pH 8, 1 mM EDTA). 4 µl of the concentrated reaction products were mixed with 4 µl of water, 1.5 µl of 500 mM Tris–HCl pH 8, 50 mM $MgCl_2$ and 1 µl of DNAse I (Sigma; 4 U/µl in 20 mM Tris–HCl pH 7.2, 1 mM $MgCl_2$). After incubation for 6 h at 37°C, 1 µl of phosphodiesterase I (Sigma, suspended in 50 mM glycine–NaOH pH 9.2 in 50% glycerol to 5 mg/ml and diluted ten-fold by 50 mM Tris–HCl pH 8, 5 mM $MgCl_2$ before use) was added. After overnight incubation at 37°C, the digested reaction products were mixed with marker nucleotides (dAMP, dCMP, dGMP, dTMP, dIMP and 5-methyl-dCMP, ~10 µg each), and extracted twice by phenol/chloroform. The aqueous phase was then separated by TLC on cellulose plates (Sigma, T-5770) developed in isobutyric acid: water: ammonium hydroxide (66: 33: 1 vol/vol) [22]. We identified the marker spots by UV illumination, and subsequently used fluorography (Enhance spray, DuPont) to detect radiolabeled nucleotides. This thin-layer chromatography assay was also used to quantify the methylation status of terminal cytosine residues generated by *Taq*I digestion of genomic DNA as described by Cedar *et al.* [22].

Measurement of S-adenosylmethionine levels

S-adenosylmethionine (Ado-met) levels were quantitated by a modification of the cation-exchange HPLC method described by Hoffman [23]. The chromatography apparatus included two Rannin HPLX pumps with a Dynamax mixer, a Rheodyne injector, a Knauer model 87 detector and Rannin software for the Macintosh. A 4.6 × 250 mm Xpertek SCX column (5 µm; P.J.Cobert, #964057), outfitted with a Brownlee cation guard column (P.J.Cobert, #933010), was used. Buffer A was made by adding 0.7 ml of concentrated NH_4OH to 1 l of 20% acetonitrile in water, and then adjusting the pH to 3.0 with 88% formic acid. Buffer B was made by bringing buffer A to 200 mM $(NH_4)_2SO_4$ (SigmaUltra A2939) and using sulfuric acid to adjust the pH to 3.0. The following elution profile was utilized: 10 ml of 20% B for the initial 5 min, a 20 ml linear gradient from 20% to 50% B, followed by 50% B for the duration of the elution. The absorbance was monitored at 258 nm and the flow rate was 2 ml/min. For the absorption profile shown in Figure 5E, elution profiles of extracts and Ado-met standards (Sigma A4377) were monitored at variable wavelengths.

To prepare extracts, 27–44 day old plants were ground in liquid nitrogen, weighed and homogenized using a polytron (Brinkmann) in 4 vol of 5% perchloric acid. The homogenate was centrifuged at 16,000 × *g* for 10 min at 4°C and the supernatant was filtered through a 0.2-µm nitrocellulose syringe filter (Supor Acrodisc 25, Gelman Science). Aliquots of the extracts were stored at –20°C until use. 200 µl of each sample were injected per chromatography run. Ado-met concentrations in the extracts were determined by reference to standard curves generated by chromatography of serial dilutions of a commercial Ado-met preparation.

RESULTS

Phenotypic analysis of *ddm1* homozygotes

We examined four phenotypic characters in two independent, segregating populations of *A.thaliana* individuals generated by self pollination of a *ddm1-2/+* heterozygote. Each population consisted of approximately 90 individuals. The characters measured included days to flowering onset, number of rosette and cauline (aerial) leaves at flowering onset, and leaf shape. After character measurements were complete, genomic DNA samples were prepared from all plants, and *ddm1/ddm1* homozygotes and non-mutants (*ddm1-2/+* and +/+) were identified by Southern analysis (for example, see Figure 1).

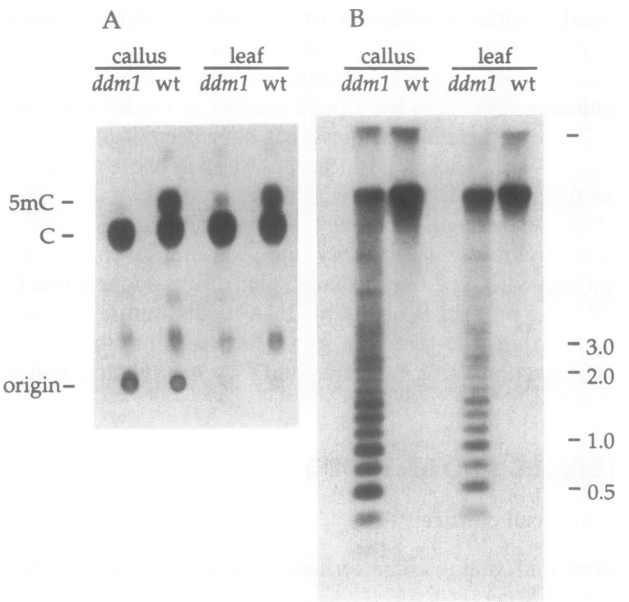

Figure 1. DNA hypomethylation in *ddm1* mutant callus. (**A**) Cytosine methylation of genomic DNA prepared from wild-type *A.thaliana* Columbia (wt) or *ddm1-2/ddm1-2* mutant callus or leaf tissue was measure in two different assays. (A) Methylation at *Taq*I sites (T/CGA). Genomic DNA was digested by *Taq*I, which cuts both methylated and unmethylated TCGA sites. *Taq*I-digested DNA was then end-labeled and digested to dNMPs. The labeled terminal cytosines were separated by thin-layer chromatography. C, dCMP; 5mC, 5-methyl-dCMP. (**B**) Cytosine methylation of *Hpa*II sites (CCGG) in centromere repeats. Genomic DNA was digested with the methylation-sensitive endonuclease *Hpa*II, size-fractionated by electrophoresis through a 1% agarose gel, and transferred to a nylon membrane. A radiolabeled 180-bp centromere repeat (pARR20-1 insert) was used as a hybridization probe. The strong 'ladder' signals seen in the *ddm1* mutant lanes indicated hypomethylation of CCGG sites in the tandem repeat arrays. Size markers are shown on the right in kilobase pairs; the top marker indicates the position of the wells.

Casual inspection of the mutants revealed no gross abnormalities. Quantitative comparison of *ddm1/ddm1* mutants versus non-mutants indicated no significant differences in the number of rosette leaves present at the onset of flowering (data not shown). However, we noted differences between the *ddm1* homozygotes and their non-mutant siblings in the other phenotypic characters. Table 1 shows representative data from one of the families examined. The mean number of cauline leaves was higher in the *ddm1* homozygotes compared to non-mutant siblings. The mean flowering time in the *ddm1* homozygotes was delayed by ~2 days relative to non-mutant siblings. In addition, the rosette leaves of *ddm1* homozygotes were rounder in shape when compared to those of non-mutant siblings.

Table 1. Phenotypic analysis of *ddm1* homozygotes and non-mutant siblings

Genotype		Phenotypic character		
	N[a]	Cauline leaf no.	Mean flower onset[b]	Leaf shape index[c]
ddm1-2/ddm1-2	22	3.27 (S.E. 0.13)	22.9 days (S.E. 0.24)	1.14 (S.E. 0.011)
Non-mutant	66	2.65 (S.E. 0.065)	21.2 days (S.E. 0.19)	1.33 (S.E. 0.013)
		P^d<0.001	P<0.001	P<0.001

[a]Sample number.
[b]Bolt (flowering stem) ≥ 1 cm.
[c]Area/width^2; lower index = rounder leaves; sixth rosette leaf measured at flowering onset.
[d]Probability that the means of the mutant and non-mutant populations are equal; two-tailed *t*-test.

Detection of *A.thaliana* DNA methyltransferase activity

To determine the mechanism by which the *ddm1* mutations cause DNA hypomethylation, we first investigated the possibility that the mutations affected DNA methyltransferase activity. In order to compare DNA methyltransferase activity between the *ddm1* mutants and the parental wild-type strain, Columbia, we developed methods to measure the enzyme activity in *A.thaliana*. Detection of plant DNA methyltransferase activities has been reported in rapidly dividing cells, such as pea shoot tip [19], cultured rice cells [21] and germinating wheat embryos [24]. Consequently, we first used cultured *A. thaliana* callus as a source of rapidly dividing cells. Hypomethylation of cytosine residues in genomic DNA prepared from *ddm1* callus cultures was comparable to that seen in DNA isolated from *ddm1* plants (See Figure 1A, B) indicating that callus is an appropriate source of material for our experiments. The confirmation of DNA hypomethylation in callus cells is also consistent with our previous observation that the reduction in DNA methylation caused by *ddm1* mutations is not tissue-specific [1].

DNA methyltransferase activity was assayed by measuring the transfer of a radiolabeled methyl group from Ado–met to exogenous substrate DNA. Activity was detected in isolated nuclei, as well as NaCl soluble nuclear extracts (Figure 2A shows the results from a representative experiment). Approximately 40–60% of the DNA methyltransferase activity was solubilized from the nuclei using 200 mM NaCl. Without addition of exogenous DNA, the NaCl insoluble fraction displayed some incorporation, presumably reflecting incorporation into endogenous DNA. When exogenous DNA was omitted, the NaCl solubilized fraction showed little or no incorporation, consistent with the expectation that the NaCl extract is free of endogenous DNA substrate. With the addition of exogenous DNA, the NaCl solubilized fractions incorporated radiolabeled methyl groups from Ado–met with linear kinetics for more than 6 h, whereas the activity of the NaCl insoluble nuclei fraction decreased after 6 h of incubation (Figure 2A). The activity in the NaCl solubilized extracts was directly proportional to the protein concentration of the extract up to at least 200 μg/ml (Figure 2B). Based on these pilot experiments, we used NaCl solubilized nuclear extracts and measured methyl group incorporation after 6 h, unless otherwise noted.

DNA methyltransferase activity in extracts of wild-type and *ddm1* mutant tissues

In order to test the hypothesis that the *DDM1* locus encodes a DNA methyltransferase, we compared DNA methyltransferase activity from *ddm1* mutant and wild-type callus cell nuclear extracts. Table 2 shows the results from ten independent experiments using the hemimethylated poly [d(CI)] substrate. In each experiment, the *ddm1* mutant nuclear extracts contained DNA methyltransferase activities comparable to, or slightly higher than, activities measured from wild-type nuclear extracts. The solubilization efficiency of the methyltransferase activity from nuclei (activity in NaCl soluble extract/[total activity in NaCl soluble and insoluble extracts]) was similar in the *ddm1* mutant and wild-type extract preparations (data not shown). The variation in absolute specific activities of the NaCl extracts from one experiment to another most likely reflects variation in preparation scale, callus age and the commercial preparation of S-adenosylmethionine used. Comparable levels of DNA methyltransferase activity were also found in extracts prepared from *ddm1* mutant and wild-type seedling tissue using hemimethylated poly [d(CI)] substrates, confirming the validity of the results obtained using callus cell extracts (data not shown).

Table 2. Specific activities of DNA methyltransferase in nuclear extracts of wild-type and *ddm1* mutant callus

	Specific activity (cpm/h/μg protein)[a]		
Experiment[b]	wild-type	*ddm1*	*ddm1*/wild-type[c]
1	14.2	15.1	1.06
2	13.1	11.2	0.85
3	8.8	11.5	1.31
4	26.5	46.1	1.74
5	12.5	24.2	1.94
6	12.7	19.3	1.52
7	38.8	51.5	1.33
8	22.5	28.3	1.26
9	22.2	20.8	0.94
10	25.0	24.0	0.96
Mean ± SD			1.29 ± 0.36

[a]DNA methyltransferase activity was measured in nuclear extracts of wild-type Columbia and *ddm1* mutant callus using hemimethylated poly [d(CI)] substrates. Mutant and wild-type extracts were prepared and assayed in parallel.
[b]Each experiment represents independent enzyme preparation and assay.
[c]The right column shows the specific activity of the *ddm1* mutant extracts normalized to wild-type for each experiment. The mean and sample standard deviation for these ratios are indicated at the base of the right column.

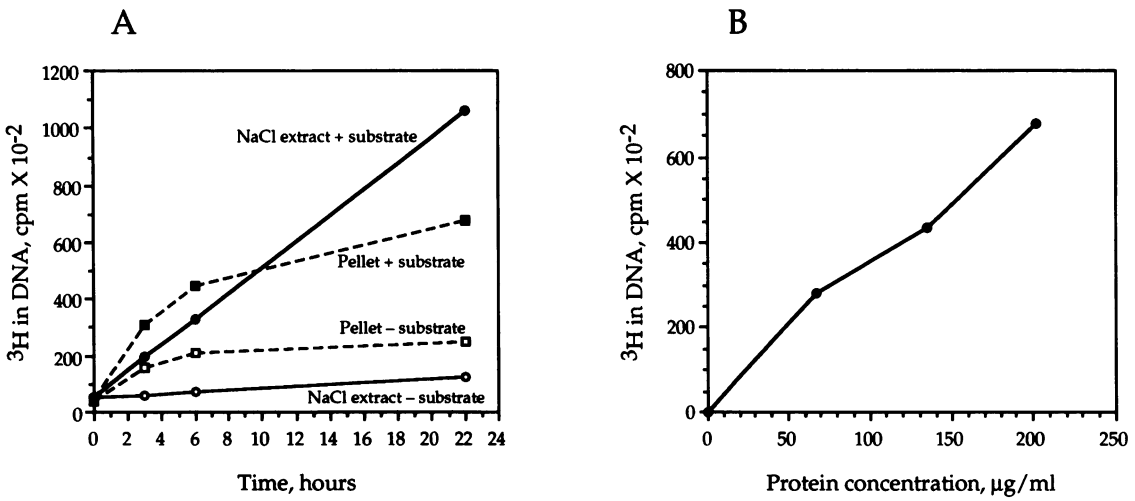

Figure 2. DNA methyltransferase activity from wild-type *A.thaliana* Columbia callus nuclei. (**A**) DNA methyltransferase activity of nuclear extracts was assayed by transfer of radiolabeled methyl groups from ^{3}H-Ado-met to alkali-resistant nucleic acid. Incorporation at various times of incubation with NaCl solubilized nuclear fractions is indicated by the circles (solid line), and incorporation for the remaining nuclear pellet is denoted by the square symbols (dashed line). Control reactions without addition of hemimethylated poly[d(CI)] substrate are indicated by open symbols. Reactions with added hemimethylated poly[d(CI)] substrate are indicated by solid symbols. Activity was normalized to protein concentration in the NaCl solubilized fractions. (**B**) The effect of increasing protein concentration of NaCl solubilized fractions on the DNA methyltransferase activity was measured. Reactions were incubated for 6 h and hemimethylated poly[d(CI)] substrates were used. Incorporation was calculated by subtracting background incorporation for parallel reactions lacking substrate DNA.

To determine if the measured activity is in fact cytosine methyltransferase, reaction products were digested to mononucleotides and analyzed by thin-layer chromatography to identify the labeled product(s). Figure 3 shows that the radioactivity was incorporated exclusively into 5-methylcytosine in both the wild-type and *ddm1* mutant reaction products.

Because cytosine methylation occurs at CpNpG sites in plants, we also measured DNA methyltransferase activity for hemimethylated CpNpG substrates. Comparable methylation of hemimethylated CpNpG substrates could be detected in *ddm1* mutant and wild-type callus cell nuclear extracts (See Figure 4).

Substrate specificity of *A.thaliana* DNA methyltransferase activity

Substrate specificities of *A.thaliana* wild-type and the *ddm1* mutant DNA methyltransferase activity were examined (Figure 4). In the *A.thaliana* callus extracts, *de novo* methyltransferase activities for both CpI and CpNpG substrates were very low, ~ 12-fold less than the activities seen for the cognate hemimethylated substrates. Methyltransferase activity in the *A.thaliana* extracts appeared to prefer the hemimethylated CpI substrate over the hemimethylated CpNpG DNA. In a parallel control experiment using nuclear extracts from pea nuclei, we detected a preference for hemimethylated substrates and a relatively high activity for the CpNpG substrates, consistent with the results of Houlston *et al.* [20].

Level of S-adenosylmethionine in wild-type and *ddm1* mutant tissues

S-adenosylmethionine (Ado-met) is the methyl group donor for DNA methyltransferase. In order to see if the hypomethylation phenotype of the *ddm1* mutants is caused by a reduction in the

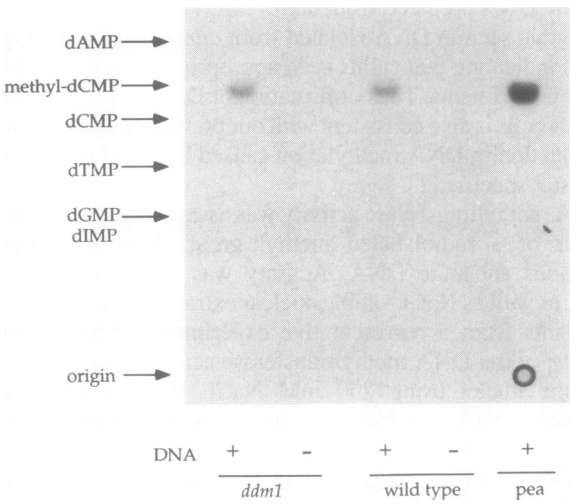

Figure 3. Nucleotide specificity of DNA methyltransferase activity. NaCl solubilized nuclear extracts from pea plants and *A.thaliana* Columbia wild-type or *ddm1/ddm1* callus cultures were incubated with hemimethylated poly[d(CI)] and ^{3}H-Ado-met for 6 h. The reaction products were digested to dNMPs and separated by thin-layer chromatography. The labeled nucleotides were visualized by fluorography.

Ado-met pool size, we compared the Ado-met levels in wild-type and *ddm1* mutant plants. Total cell extracts from *ddm1* mutant and wild-type Columbia plant tissue were analyzed by cation-exchange HPLC (see Figure 5 for a representative experiment). Data from three independent Ado-met quantitation experiments are tabulated in Table 3. Ado-met levels in the *ddm1* mutants are indistinguishable from those of the wild-type plants; both the *ddm1* plants and the wild-type plants have between 3 to 5 µg of Ado-met per gram of fresh weight.

Table 3. S-adenosyl-methionine levels in extracts of wild-type and *ddm1* mutants

Genotype	Experiment[a]			Mean (S.D.)
	One	Two	Three	
ddm1-2/ddm1-2	4.18 (S.D. 0.01)	4.45 (S.D. 0.01)	3.70 (S.D. 0.03)	4.11 (S.D. 0.38)
Wild-type	4.84 (S.D. 0.13)	4.18 (S.D. 0.02)	3.01 (S.D. 0.10)	4.01 (S.D. 0.93)

[a]Each experiment represents independent extract preparation and assay.

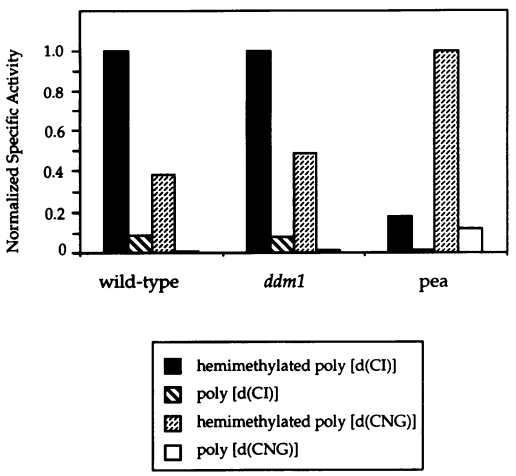

Figure 4. Substrate specificity of DNA methyltransferase activity. NaCl solubilized nuclear extracts from pea plants and *A. thaliana* Columbia wild-type or *ddm1/ddm1* callus cultures were incubated with hemimethylated poly[d(CI)] and ^{3}H-Ado-met for 6 h. Incorporation was calculated by subtracting background incorporation for parallel reactions lacking substrate DNA. The activities were normalized to the activity seen using the most efficient substrate for each set of reactions. The specific activities for DNA methyltransferase in the wild-type, *ddm1* mutant, and pea extracts using hemimethylated poly [d(CI)] substrates were 12.7, 19.3, and 395 cpm/h/μg protein, respectively.

DISCUSSION

We have extended our characterization of an *A.thaliana* DNA hypomethylation mutant, by examining both the phenotype of *ddm1* mutants and the possible biochemical basis of the DNA hypomethylation. Our phenotypic analysis confirms our earlier report that *ddm1/ddm1* homozygotes do not display striking morphological abnormalities. However, the mutants exhibited significant alterations in cauline leaf number, flowering time, and rosette leaf shape. Traits such as leaf shape and flowering time are complex characters, and the connection between these traits and DNA hypomethylation is not presently understood. A model consistent with current understanding is that DNA hypomethylation leads to aberrant gene expression, and subsequently, altered development.

We stress that more phenotypic analysis of *ddm1* mutants remains to be done. We have seen a suite of more dramatic morphological phenotypes appearing in *ddm1* mutant lines that were generated by repeated self-pollination of homozygous parents [25]. The nature of these phenotypic changes is currently under investigation.

Our biochemical characterization focused on two possible mechanisms for the DNA hypomethylation phenotype of *ddm1* mutants. We first investigated possible effects on DNA methyltransferase activity. DNA methyltransferase activity from *ddm1* mutant callus was as high as that of the wild-type for both CpG and CpNpG substrates. These results indicate that the hypomethylation phenotype of *ddm1* mutation is not due to the loss of DNA methyltransferase function.

The DNA methyltransferase activity detected in the *A.thaliana* extracts has the characteristic of preferring hemimethylated substrates over their unmethylated cognates. A similar preference has been described for a variety of plant and mammalian DNA methyltransferases [10, 20, 26] and probably reflects the 'maintenance' methylation activity that ensures clonal propagation of the genomic methylation patterns through mitotic divisions during development [27, 28]. The specific activity of DNA methyltransferase in the *A.thaliana* extracts was less than 5% of that measured in pea extracts. The relatively low specific DNA methyltransferase activity in the *A.thaliana* extracts correlates with the low DNA methylation content in *A.thaliana*. [29, 30].

It should be noted that our assay primarily detects maintenance methylation activity in both the wild-type and the *ddm1* mutant *A.thaliana* extracts. We believe that the hypomethylation of the mutant is not likely to be due to loss of a *de novo* DNA methyltransferase activity, which adds methyl groups to an unmethylated substrate, because no evidence exists for global demethylation and remethylation of the genome during the plant life cycle [31, 32]. Moreover, our previous results demonstrate a lack of appreciable *de novo* methylation during the life cycle of wild-type *A.thaliana* [1].

A candidate *A.thaliana* DNA methyltransferase gene has recently been cloned from a cDNA library based on homology between prokaryotic and mouse methyltransferases [33]. RFLPs recognized by this gene did not co-segregate with the *ddm1* mutations [25]. Although additional DNA methyltransferase genes may be identified in the future, the available genetic mapping data and the biochemical results presented here argue that the *DDM1* locus does not encode a DNA methyltransferase.

We also investigated the possibility that the DNA hypomethylation phenotype of *ddm1* mutants may result from a reduction in cellular levels of Ado-met, the methyl group donor for a variety of transmethylation reactions. Total cell extracts of *ddm1* mutant and wild-type plant tissues contain indistinguishable amounts of Ado-met. There is no evidence for a separate, minor nuclear pool of Ado-met in plant cells, and the molecular weight of Ado-met is far below the diffusion threshold of nuclear pores [34]. Consequently, we believe that a reduction in intranuclear Ado-met concentrations is not likely to be responsible for the *ddm1* DNA hypomethylation phenotypes.

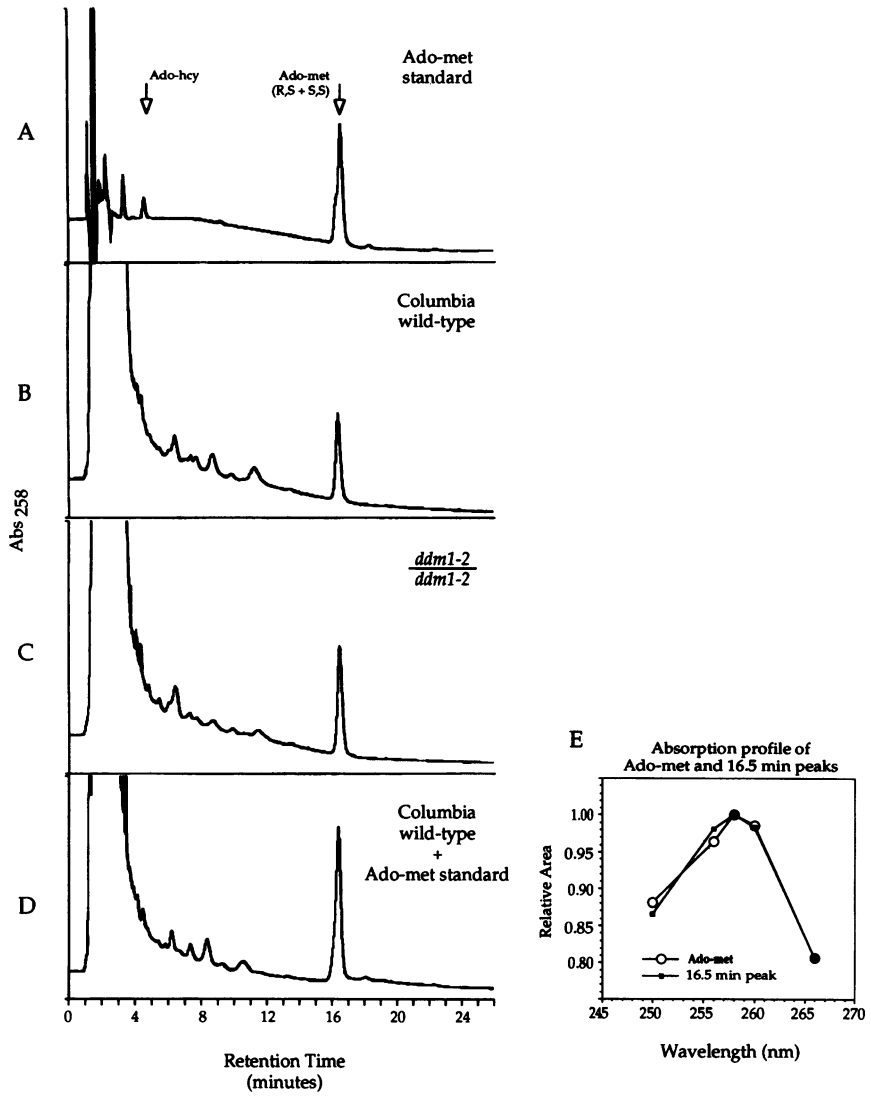

Figure 5. Cellular Ado–met levels in wild-type and *ddm1* mutant *A. thaliana* plants were determined by cation-exchange HPLC analysis of whole cell extracts. (**A**) Chromatogram of Ado-met standard; two stereoisomers of Ado-met (R,S and S,S) are indicated, as well as S-adenosylhomocysteine (Ado-hcy). A peak displaying a retention time 16.5 min was seen in the elution profiles of extracts prepared from wild-type Columbia (**B**), and *ddm1* mutant (**C**) plants. This peak co-elutes with the Ado-met standard (**D**). The absorption profile of the 16.5 min peak from the extracts matches that of the Ado-met standard (**E**).

Our results indicate that *ddm1* mutations do not reduce cellular DNA methyltransferase activity nor Ado-met levels. Because the enzymatic machinery responsible for DNA methylation is present in the *ddm1* mutants, we suggest that the DNA hypomethylation phenotype of the mutants stems from alteration in the regulation or intracellular context of the DNA methylation reaction.

One possibility is that the *DDM1* gene product serves as a regulator of DNA methyltransferase. Functional domains of mammalian DNA methyltransferase have recently been found that may interact with other factors to establish DNA methylation patterns. A domain in the amino terminal portion of the murine enzyme functions as a signal for cell-cycle dependent association of the methyltransferase with replication foci [35]. The intracellular localization of the murine DNA methyltransferase is also developmentally regulated [12]. Possibly, the *DDM1* gene product could be a component of the machinery that directs DNA methyltransferase localization.

If not functioning in direct association with the DNA methyltransferase, the *DDM1* gene product might affect DNA methylation indirectly by changing the intracellular context of DNA methylation. For example, *ddm1* mutations may alter chromosomal structure in such a way to preclude methylation of certain chromosome domains. Alternatively, a shift in DNA replication timing or replicon identity may cause certain classes of genomic sequences to be unavailable for inclusion in methyltransferase-rich replication foci [35].

Although we have not distinguished among these alternatives, the *DDM1* gene product is likely to be involved in DNA methylation at a fundamental level, because *ddm1* mutations affect the methylation of a wide variety of genomic sequences [1]. In this respect, the *ddm1* mutations differ from other modifiers of DNA methylation found in mouse, which are known to regulate methylation of specific transgenes, but do not affect global levels of DNA methylation [14]. Cloning and characterization of the

DDM1 gene should clarify these points and help elucidate the mechanisms regulating methylation of the eukaryotic genome.

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