**RESEARCH COMMUNICATION**

**Arabidopsis cmt3 chromomethylase mutations block non-CG methylation and silencing of an endogenous gene**

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Plants maintain cytosine methylation at CG and non-CG residues to control gene expression and genome stability. In a screen for *Arabidopsis* mutants that alter methylation and silencing of a densely methylated endogenous reporter gene, we recovered 11 loss-of-function alleles in the *CMT3* chromomethylase gene. The *cmt3* mutants displayed enhanced expression and reduced methylation of the reporter, particularly at non-CG cytosines. CNG methylation was also reduced at repetitive centromeric sequences. Thus, *CMT3* is a key determinant for non-CG methylation. The lack of *CMT* homologs in animal genomes could account for the observation that in contrast to plants, animals maintain primarily CG methylation.

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In many eukaryotes, including mammals, higher plants, and some species of fungi, cytosine methylation plays an important role in genome stability and development by altering chromatin structure and patterns of gene expression. In mammalian genomes, methylation is found primarily at cytosines in the symmetric context 5’-CG-3’ (CG), whereas in plant and fungal genomes methylation is found on both CG and non-CG residues (Yoder et al. 1997; Colot and Rossignol 1999; Finnegan and Kovac 2000). Mammals and higher plants carry related cytosine methyltransferases of the Dnmt1/MET1 class that have been implicated by mutational analysis as enzymes that maintain the bulk of genomic methylation (Li et al. 1997; Colot and Rossignol 1999; Finnegan and Kovac 2000). Recently, *Arabidopsis* CMT2 (Lindroth et al. 2001) and the maize CMT homolog ZMET2 (Papa et al. 2001) have been implicated in the maintenance of CNG methylation.

The genome of *Arabidopsis*, duplicated genes encoding the tryptophan pathway enzyme phosphoribosylanthranilate isomerase (PAI) provide a well-characterized example of endogenous genes that are densely methylated with both CG and non-CG methylation (Luff et al. 1999). In the *Arabidopsis* strain WS, there are four methylated PAI genes at three unlinked loci: a singlet PAI2 gene that encodes functional enzyme, a singlet PAI3 gene that does not encode functional enzyme, and a tail-to-tail inverted repeat of the PAI1 and PAI4 genes [PAI1-PAI4]) in which the PAI1 gene encodes functional enzyme and the PAI4 gene does not (Bender and Fink 1995; Melquist et al. 1999). The functional singlet PAI2 gene is silenced by methylation (Bender and Fink 1995; Jeddelah et al. 1998; Melquist et al. 1999). In contrast, the functional PAI1 gene in the inverted repeat is expressed despite dense methylation in the body of the gene, providing sufficient PAI enzyme for a wild-type plant phenotype (Melquist et al. 1999). It is likely that the WS PAI1 gene eludes silencing by methylation because of novel promoter sequences lying upstream of the methylated region (Melquist et al. 1999; J. Bender, unpubl.).

Here we describe the isolation and characterization of mutations in the *CMT3* chromomethylase gene from a genetic screen for reduced PAI methylation. Southern blot analysis and bisulfite genomic methylation sequencing indicate that *cmt3* mutations confer a partial loss of CG methylation and a strong loss of non-CG methylation (both CNG and asymmetric cytosines) from the PAI genes. Southern blot analysis of repetitive methylated genomic sequences indicates that *cmt3* mutations also confer reduced CNG methylation on these regions. In contrast to characterized *Arabidopsis* mutations that confer globally decreased methylation (Finnegan et al. 1996; Kakutani et al. 1996; Ronemus et al. 1996), the *cmt3* mutations do not lead to pleiotropic effects upon inbreeding, suggesting that CMT3 function is specialized for only a subset of methylated regions in the genome.

**Results and Discussion**

To identify factors that control methylation and silencing of the WS PAI genes, we isolated a mutant variant of WS, *pai1C251Y*, in which silencing of the methylated singlet PAI2 gene can be visualized by the intensity of a blue fluorescent plant phenotype under ultra-violet (UV) light (Bartee and Bender 2001). In the *pai1C251Y* strain, the only potential sources of PAI enzyme activity are the PAI1 gene, which is crippled by a missense mutation, and CMT3 (Henikoff and Comai 1998; Finnegan and Kovac 2000; McCallum et al. 2000). In the Wassilewskija (WS) strain background used for this study, CMT2 and CMT3 are predicted to encode functional proteins, whereas the *CMT1* coding sequence is disrupted by an *Eve1* [Henikoff and Comai 1998] retroelement insertion [J. Bender, unpubl.]. *CMT* genes have also been identified in several other plant species including *Brassica* and maize, but not in fungal or animal systems (Rose et al. 1998; Finnegan and Kovac 2000). Recently, *Arabidopsis* CMT3 (Lindroth et al. 2001) and the maize CMT homolog ZMET2 (Papa et al. 2001) have been implicated in the maintenance of CNG methylation.

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and the PAI2 gene, which is silenced. Because of this PAI deficiency, the strain accumulates fluorescent tryptophan pathway intermediates, as well as displaying yellow-green leaf pigmentation, reduced size, increased bushiness, and reduced fertility. However, second-site mutations that relieve PAI2 silencing will suppress the PAI-deficient phenotypes (Bartee and Bender 2001). Thus, we mutagenized the pai1C251Y strain and screened for seedlings with suppressed weak fluorescent phenotypes. As a secondary screen, we tested PAI methylation by Southern blot analysis with methylation-sensitive restriction enzymes. Specifically, we assayed methylation with the isoschizomers HpaII and MspI, which recognize the sequence 5′-CCGG-3′. HpaII is sensitive to methylation of both the inner [CG] and the outer [CNG] cytosines, whereas MspI is only sensitive to methylation of the outer cytosines. These enzymes cleave once in each WS PAI locus and reveal both the density and the pattern of methylation for each gene (Bender and Fink 1995; Luff et al. 1999, Melquist et al. 1999).

From this screening strategy we isolated 11 loss-of-function alleles in the CMT3 gene (see below and Materials and Methods). The cmt3 mutants in the pai1C251Y background displayed strongly reduced fluorescence in early seedling development and partially reduced fluorescence in adult plants, with increased size, decreased bushiness, and increased fertility (Fig. 1). These intermediate fluorescent cmt3 isolates did not revert to nonfluorescence, which is diagnostic of loss of residual PAI2 methylation (Bender and Fink 1995), at a detectable frequency. They displayed partially increased cleavage with HpaII and strongly increased cleavage with MspI for the PAI genes relative to parental pai1C251Y (Fig. 2A). The cleavage pattern suggested that the cmt3 mutants were...
most affected in maintenance of CNG methylation of the PAI genes. To determine whether the cmt3 mutants also affected methylation of a highly repeated genomic sequence, we reprobed the HpaII/MspI Southern blot with a probe to the 180-bp centromere-associated repeat (CEN) sequences (Vongs et al. 1993). This probe revealed little effect on HpaII cleavage but increased MspI cleavage, consistent with the pattern observed for the PAI genes (Fig. 2B). A similar pattern of increased MspI cleavage was also observed at the repeated rDNA (data not shown). All of 11 cmt3 alleles tested had identical methylation patterns in these assays. Moreover, when the cmt3 alleles were segregated away from the pai1C251Y allele into a wild-type WS background, they also displayed identical methylation patterns in these assays (Fig. 2). The PAI and CEN methylation patterns were distinct from the patterns induced by the characterized ddm1 and met1 methylation-deficient mutations (Fig. 2; Barbee and Bender 2001).

To more precisely determine methylation patterns in the cmt3 mutant background, we performed genomic sequencing of methylation patterns in the PAI1 and PAI2 promoter regions of a representative cmt3 allele by using sodium bisulfite mutagenesis (Frommer et al. 1992). This analysis revealed that the majority of methylated cytosines (87% in PAI1 and 70% in PAI2) occurred at CG residues (Fig. 3; Table 1). Compared with the wild-type WS PAI1 promoter (Luff et al. 1999; Table 1), CG methylation was reduced 34%, CNG methylation was eliminated, and asymmetric methylation was reduced 93%; in the PAI2 promoter, CG methylation was reduced 8%, CNG methylation was reduced 92%, and non-CG methylation was reduced 75%. Thus, loss of CMT3 function has a strong effect on maintenance of CNG and asymmetric methylation and a weaker effect on maintenance of CG methylation. These results are consistent with reports that Arabidopsis CMT3 and maize ZMET2 are important for maintenance of CNG methylation at various genomic sites (Lindroth et al. 2001; Papa et al. 2001), but they further show that CMT3 is also important for maintenance of asymmetric methylation for the PAI genes. This result implies either that CMT3 directly controls both symmetric and asymmetric methylation or that the reduction in symmetric methylation in the cmt3 mutant background causes reduced asymmetric methylation as a secondary consequence. Because the methylated sequences in the promoter and first exon of the PAI2 reporter gene (~370 bp) contain only 16 dispersed CG motifs, loss of non-CG methylation significantly hypomethylates this region of the gene (Fig. 3), accounting for enhanced PAI2 expression in the suppressor mutant.

The cmt3 mutant locus in the pai1C251Y cmt3 suppressor isolates was mapped by crosses with the polymorphic strain Nd-0, which has a similar arrangement of densely methylated PAI genes as found in WS (Melquist et al. 1999). F2 progeny with weakly fluorescent phenotypes diagnostic of homozygosity for both pai1C251Y and cmt3 were identified by visual inspection under UV light and confirmed by MspI Southern blot for strong PAI cleavage similar to that observed in the parental suppressor isolates. A mapping population of F2 plants that fulfilled these criteria was then used to score for genomic loci linked to the suppressed phenotype. The mapping analysis revealed linkage to a single locus on the lower arm of chromosome 1. Because the CMT3 putative cytosine methyltransferase

![Figure 3](image-url)  
Figure 3. Sequencing of PAI promoter methylation in the cmt3 mutant. Bisulfite genomic methylation sequencing was performed as described (Jeddlel et al. 1998; Luff et al. 1999) for the top strands of the PAI1 and PAI2 promoters in Wassilewskija (WS) pai1C251Y cmt3G456D DNA. For each region, eight independent molecules were sequenced. Vertical lines indicate positions of cytosines, with the height of each line representing how many sequenced molecules had 5-methyl-cytosine. (Black) CG cytosines, (blue) CNG cytosines, (red) other cytosines. Asterisks indicate sites with no methylation. The black horizontal line indicates the region of PAI1 identity, and the gray horizontal line indicates flanking upstream heterologous sequence unique to each gene. The exon and intron structures of PAI1 and PAI2 are shown as open boxes and dashed lines, respectively, under each sequence. These structures are based on full-length cDNA sequences for each gene (Melquist et al. 1999).

| Strain | PAI  | CG   | CNG  | Other C | Total C |
|-------|------|------|------|---------|---------|
| WS    | PAI1 | 115 (100%) | 61 (100%) | 149 (100%) | 325 (100%) |
|       | PAI2 | 122 (100%) | 53 (100%) | 184 (100%) | 359 (100%) |
| cmt3  | PAI1 | 76 (66%) | 0 (0%) | 11 (7%) | 87 (27%) |
|       | PAI2 | 112 (92%) | 4 (8%) | 45 (25%) | 161 (45%) |

*The numbers of methylated cytosines in the indicated sequence contexts for eight independent top strand bisulfite sequencing clones for the indicated PAI promoter regions of the indicated strains are shown. The wild-type Wassilewskija (WS) data are derived from data published previously in Luff et al. (1999). The cmt3 data are from this work, and are shown in diagram form in Figure 3.

*The sequenced DNA was from the WS pai1C251Y cmt3G456D strain.
gene maps to this locus, we focused on this gene as a candidate. Within each mapping population, we found complete linkage to a polymorphic marker that lies within 100 kb of the CMT3 gene. To confirm that the CMT3 gene was in fact the site of the methylation suppressor mutations, we cloned and sequenced the gene from the 11 mutant isolates. Sequencing revealed a single base change in the CMT3 coding sequence in each isolate. Three of the mutant alleles affected absolutely conserved amino acids in the methyltransferase catalytic domain, including the representative cmt3G456D allele used for bisulfite sequencing. Another allele was predicted to prematurely terminate the protein. Two alleles created splice junction mutations. The remaining five alleles affected amino acids between methyltransferase motif IV and the C terminus of the protein that are highly conserved among the CMT genes (Fig. 4).

To further confirm that the CMT3 gene was the mutant locus, we transformed the pai1C251Y cmt3 isolates with a wild-type WS genomic clone of the CMT3 gene. Transformant seedlings were similarly fluorescent, similarly to those of the pai1C251Y strain (Fig. 1). Transformant lines assayed by Southern blot analysis in the T2 generation showed remethylation of the PAI2 gene to the levels observed in the original pai1C251Y strain (data not shown). Thus, the cloned CMT3 gene could complement the mutant methylation defects. As a control, the representative pai1C251Y cmt3G456D mutant was also transformed with a wild-type WS genomic clone of the CMT2 gene. CMT2 transformant seedlings were weakly fluorescent, similarly to those of the untransformed parental strain (Fig. 1), and did not display detectable remethylation. This analysis shows that CMT2 cannot substitute for CMT3 function.

In this regard, it is interesting to note that CMT2 differs from CMT3 primarily in its N-terminal sequence (Fig. 4). Previously characterized methylation-deficient Arabidopsis strains with defects in either the SWI2/SNF2 chromatin remodeling factor-related gene DDM1 [Jeddeloh et al. 1999] or the Dnmt1-related MET1 cytosine methyltransferase gene display progressive developmental abnormalities [Finnegan et al. 1996; Kakutani et al. 1996; Ronemus et al. 1996]. Our preliminary analysis of six-generation-inbred pai1C251Y cmt3 and two-generation-inbred cmt3 strains revealed no obvious segregation of morphological changes. This difference between cmt3 and other methylation-deficient mutants is likely to reflect the fact that CG methylation is retained to a higher degree in cmt3 than in ddm1 or met1 [Fig. 2, Bartee and Bender 2001]. Because many
creased methylation density leads to increased silencing. A specific model is that the basal layer of CG methylation provided by other functions such as MET1 could serve as a guide for CMT3, which would then decorate the basal layer with extra CG and non-CG methylation. CMT3 recruitment to targeted regions could involve chromatin protein interactions with the chromodomain motif [Henikoff and Comai 1998], along with interactions mediated by the unique N-terminal sequences.

Because fungi such as Neurospora crassa and Asco- bulus immersus can maintain non-CG methylation [Selker et al. 1993; Goyon et al. 1994], these organisms might encode CMT genes. Conversely, because animals such as humans and mice lack non-CG methylation, these organisms are predicted to lack CMT genes, as is the case from analyses of current sequence databases. The apparent lack of CMT-like methylases in animal genomes [Finnegan and Kovac 2000] suggests that animals have evolved alternate mechanisms for reinforcing chromatin states.

Materials and methods

Mutant isolation and sequencing

Seeds of WS pai1C251Y were mutagenized with ethylmethane sulfonate (Niyogi et al. 1993) and grown up as 20 pools of ~500 M1 plants each. M2 progeny seeds were collected from each pool. Approximately 1000 seedlings from each M2 pool were grown on agar medium for 2 wk and then screened with a hand-held short wavelength UV light source for individuals with reduced fluorescence. Putative mutants were transplanted to soil, and genomic DNA prepared from a single leaf was used for Southern blot analysis of methylation patterns. From the screen of 20,000 M2 seedlings, all 11 isolates that displayed cmt3 methylase patterns (Fig. 2) proved to be cmt3 alleles by mapping, sequencing, and complementation analysis. Ten other isolates with distinct methylation patterns as determined by HpaII/MspI Southern blot analysis were also recovered, but these isolates remain to be characterized. Interestingly, none of the other isolates displayed the PAI and CEN Southern blot phenotypes diagnostic of ddm1 mutations in the WS background (Fig. 2). Because we expect to recover ddm1 alleles from the screen [Bartee and Bender 2001], this observation suggests that the screen is not yet saturated.

The cmt3 mutant locus was mapped to the lower arm of chromosome 1 with standard CAPS [Konieczny and Ausubel 1993] and simple sequence length polymorphism (SSLP) [Bell and Ecker 1994] markers that are polymorphic between WS and Nd-0. It was further localized between the markers NsF114 and NsF22X20 [http://www.arabidopsis.org/servlets/mapper]. Linkage to CMT3 was determined with the T17F3 marker, which lies within 100 kb of the CMT3 gene: forward primer 5’-ga-acatataacagctagccaa-3’; reverse primer 5’-ccacacagctagcagcagc-3’; in WS a 354-bp product cleaves once with MspI into 240 bp and 114 bp fragments, whereas the Nd-0 product is uncleaved.

Mutant alleles of CMT3 were amplified by PCR from genomic DNA. Products from two independent PCRs were cloned and sequenced for each allele. Alleles that changed restriction sites (both splice site mutations, cmt3G456D, cmt3G465D, and cmt3R703K) were confirmed by PCR amplification of the mutant region followed by cleavage with the appropriate enzyme.

CMT3 genomic and cDNA clones

The CMT3 transgene is a WS genomic fragment extending from 2.9 kb upstream of the start codon to 0.8 kb downstream of the stop codon subcloned into the pBIN19 transformation vector [Bevan 1984]. The CMT2 transgene is a WS genomic fragment extending from 1.7 kb upstream of the start codon to 0.9 kb downstream of the stop codon subcloned into pBIN19. Both clones were isolated by hybridization from a WS genomic library [Bender and Fink 1999]. Both clones were sequenced across the coding region to confirm their structure and determine WS polymorphisms. Transgenes were introduced into the WS pai1C251Y cmt3G456D strain by an in planta transformation method [Clough and Bent 1998].

Arabidopsis CMT3 chromomethylase mutations

The CMT3 predicted amino acid sequence was determined by cloning and sequencing a cDNA isolate generated by RT-PCR from WS whole-plant RNA. CMT2 and ZMET2 amino acid sequences are predicted from the WS CMT2 genomic sequence, cDNA sequences available from the database, and alignment with related genes.

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