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. 1992; Finnegan et al. 1996; Ronemus et al. 1996). Another class of chromomethylases (CMTs) has been identified by analysis of *Arabidopsis thaliana* genomic sequences (Henikoff and Comai 1998; McCallum et al. 2000). The CMT class is characterized by the presence of a chromodomain amino acid motif between the cytosine methyltransferase catalytic motifs I and IV. There are three *CMT* genes encoded in *Arabidopsis*: *CMT1*, *CMT2*, 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.

In 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; Jeddeloh 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,

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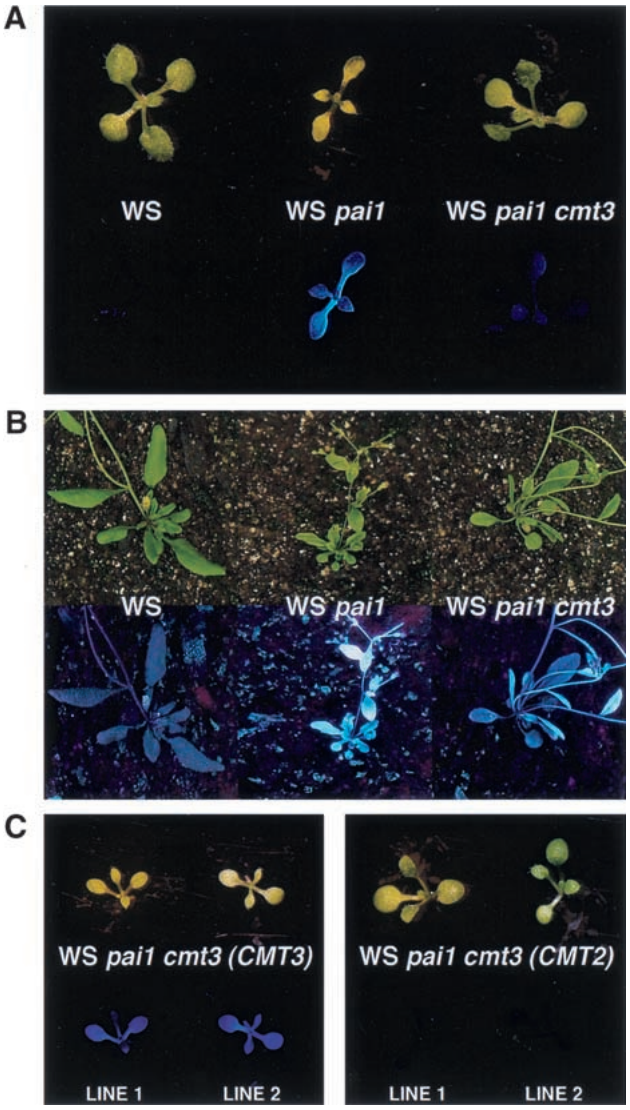

Figure 1. Wassilewskija (WS) *pai1C251Y cmt3* plant phenotypes. (*A*) Two-week-old seedlings of the indicated genotypes grown on agar medium are shown under visible (*top*) and UV (*bottom*) light. (*B*) Four-week-old adult plants of the indicated genotypes grown in soil are shown under visible (*top*) and UV (*bottom*) light. (*C*) Representative 2-week-old T2 generation transgenic seedlings of the indicated genotypes grown on agar medium are shown under visible (*top*) and UV (*bottom*) light. The phenotypes of the *cmt3G456D* allele shown are representative of the phenotypes observed with 10 other *cmt3* alleles.

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 *Hpa*II and *Msp*I, which recognize the sequence 5′-CCGG-3′. *Hpa*II is sensitive to methylation of both the inner (CG) and the outer (CNG) cytosines, whereas *Msp*I 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 *Hpa*II and strongly increased cleavage with *Msp*I for the *PAI* genes relative to parental *pai1C251Y* (Fig. 2A). The cleavage pattern suggested that the *cmt3* mutants were

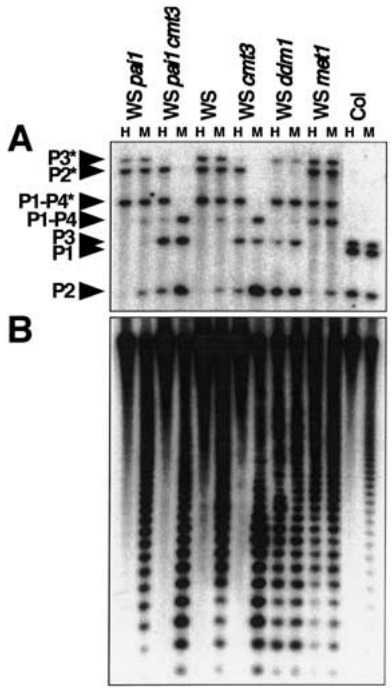

Figure 2. *cmt3* mutations confer reduced *PAI* and *CEN* methylation. (*A*) Genomic DNAs prepared from 4-week-old plants of the indicated genotypes were cleaved with either *Hpa*II (H) or *Msp*I (M) and used for Southern blot analysis with a *PAI* probe (Bender and Fink 1995). (P1–P4) *PAI1–PAI4*; (P2) *PAI2*; (P3) *PAI3*; (P1) Columbia (Col) strain *PAI1*; asterisks indicate the positions of species methylated at internal *Hpa*II/*Msp*I sites (Bender and Fink 1995; Luff et al. 1999). The Col strain is included as a control for the positions of unmethylated *PAI2* and *PAI3* species. (*B*) The blot shown in *A* was reprobed with a 180-bp *CEN* repeat probe. The phenotypes of the *cmt3G456D* allele shown are representative of the phenotypes observed with 10 other *cmt3* alleles.

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 *Hpa*II/*Msp*I Southern blot with a probe to the 180-bp centromere-associated repeat (*CEN*) sequences (Vongs et al. 1993). This probe revealed little effect on *Hpa*II cleavage but increased *Msp*I cleavage, consistent with the pattern observed for the *PAI* genes (Fig. 2B). A similar pattern of increased *Msp*I 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; Bartee 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.

Table 1. *Effects of a* cmt3 *mutation on patterns of* PAI *promoter cytosine methylation*[a]

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

[a]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.
[b]The sequenced DNA was from the WS *pai1C251Y cmt3G456D* strain.

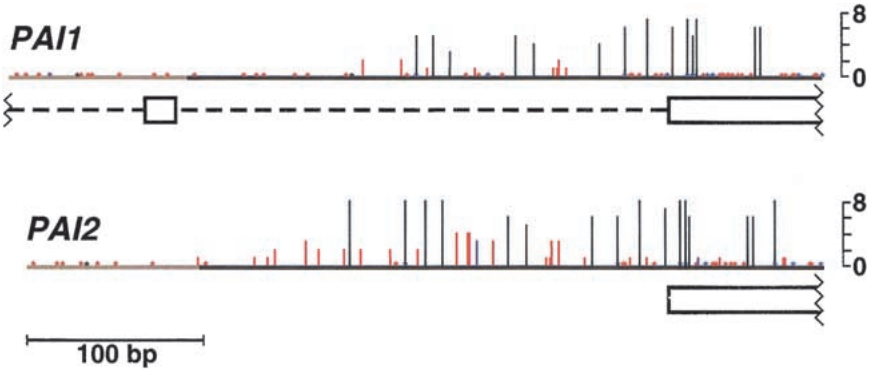

Figure 3. Sequencing of *PAI* promoter methylation in the *cmt3* mutant. Bisulfite genomic methylation sequencing was performed as described (Jeddeloh et al. 1998; Luff et al. 1999) for the top strands of the *PAI1* and *PAI2* promoters in Wassilewskija (WS) *pai1C251Y cmt3G456D* DNA. For each region, 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 *PAI* 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).

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). F_2 progeny with weakly fluorescent phenotypes diagnostic of homozygosity for both *pai1C251Y* and *cmt3* were identified by visual inspection under UV light and confirmed by *Msp*I Southern blot for strong *PAI* cleavage similar to that observed in the parental suppressor isolates. A mapping population of F_2 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

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 strongly 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 of *PAI2*. 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 of the endogenous methylated sites in the *Arabidopsis* genome, such as the *CEN* repeats (Fig. 2; Vongs et al. 1993; Lindroth et al. 2001), and the promoter of the *FWA* homeo-domain gene (Soppe et al. 2000; Lindroth et al. 2001), carry primarily CG methylation, *cmt3* mutations would not be expected to strongly affect these loci. Instead, *CMT3* most likely acts as a reinforcing methylase that adds an extra layer of methylation to particular genomic regions such as the *PAI* genes, in which the in-

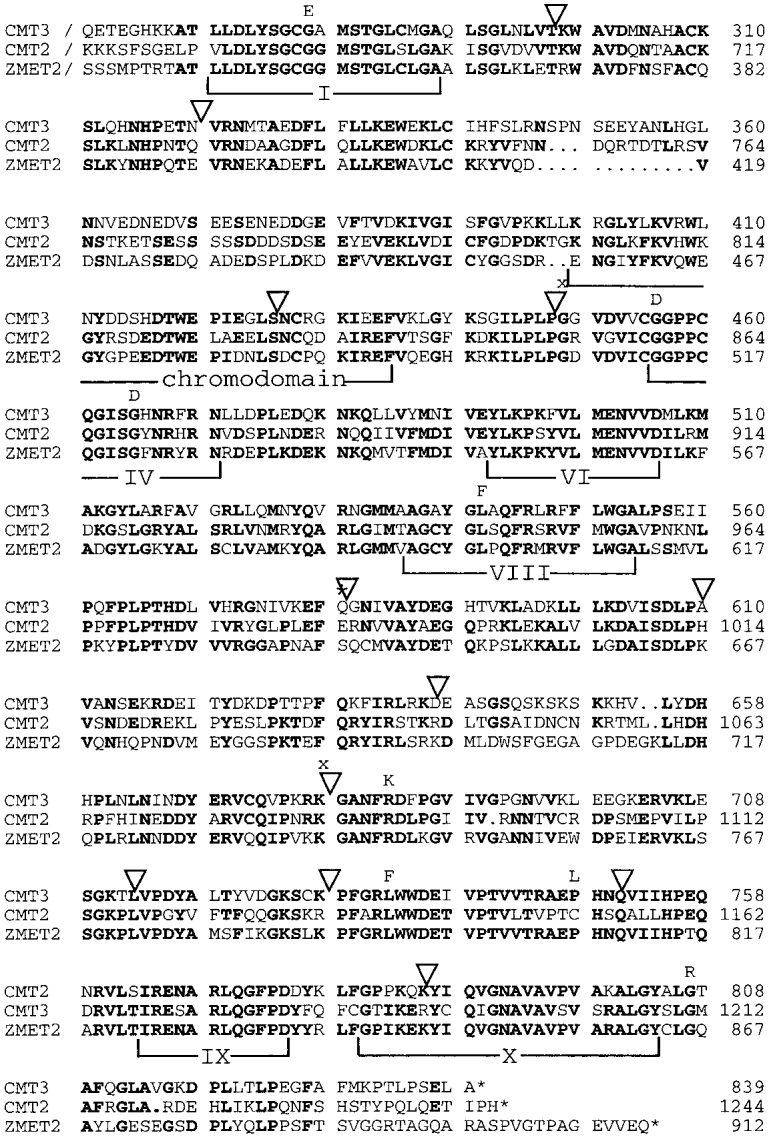

Figure 4. Positions of mutations in *CMT3*. The predicted amino acid sequences of Wassilewskiya (WS) *CMT3*, WS *CMT2*, and maize *ZMET2* are shown aligned along their conserved C-terminal regions. The N termini, upstream of the backslash at the beginning of each sequence, are unrelated. *CMT3* introns are indicated by inverted triangles above the sequence. *CMT3* missense mutations are indicated above the affected residues. The stop mutation is indicated by an asterisk, and the splice donor and acceptor site mutations are indicated by an x to the left or right, respectively, above the affected introns. Residues identical between proteins are highlighted in boldface. Conserved sequence motifs are indicated under the alignment. GenBank accession nos. are: AF383170 for WS *CMT3* and AF383171 for WS *CMT2*.

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 *Ascobolus 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* methylation patterns (Fig. 2) proved to be *cmt3* alleles by mapping, sequencing, and complementation analysis. Ten other isolates with distinct methylation patterns as determined by *Hpa*II/*Msp*I 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 NF5I14 and NF22K20 (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′-gacataatacgagtacccac-3′; reverse primer 5′-ccaccaccttgcactgccgacc-3′; in WS a 354-bp product cleaves once with *Msp*I 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.

CMT 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 1995). 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).

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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