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Site specificity of the *Arabidopsis* METI DNA methyltransferase demonstrated through hypermethylation of the *superman* locus

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Abstract

Plants with low levels of DNA methylation show a range of developmental abnormalities including homeotic transformation of floral organs. Two independent *DNA METHYLTRANSFERASEI* (*METI*) antisense transformants with low levels of DNA methylation had flowers with increased numbers of stamens which resembled flowers seen on the loss-of-function *superman* (*sup*) mutant plants and on transgenic plants that ectopically express *APETALA3* (*AP3*). These *METI* antisense plants have both increased and decreased methylation in and around the *sup* gene, compared with untransformed controls. DNA from the antisense plants was demethylated at least 4 kb upstream of the *sup* gene, while there was dense methylation around the start of transcription and within the coding region of this gene; these regions were unmethylated in control DNA. Methylation within the *sup* gene was correlated with an absence of *SUP* transcripts. The pattern and density of methylation was heterogeneous among different DNA molecules from the same plant, with some molecules being completely unmethylated. Methylcytosine occurred in asymmetric sites and in symmetric CpA/TpG but rarely in CpG dinucleotides in the antisense plants. In contrast, segregants lacking the *METI* antisense construct and epimutants with a hypermethylated allele of *sup* (*clark kent 3*), both of which have active *METI* genes, showed a higher frequency of methylation of CpG dinucleotides and of asymmetric cytosines. We conclude that METI is the predominant CpG methyltransferase and directly or indirectly affects asymmetric methylation.

Introduction

The importance of DNA methylation in plant development has been shown by examining the effects of genome-wide demethylation in *Arabidopsis*. Plants with low levels of methylation resulting from transformation with an antisense construct of *METI*, a DNA methyltransferase (Finnegan and Dennis, 1993; Finnegan *et al.*, 1996; Ronemus *et al.*, 1996), or by mutation in DDM1 (Vongs *et al.*, 1993; Kakutani *et al.*, 1995), a protein with homology to the SNF2/SWI2 family of chromatin remodelling proteins (Jeddeloh *et al.*, 1999), displayed abnormal phenotypes, including loss of apical dominance, reduced stature, altered leaf size and shape, altered root architecture, abnormal floral development and altered flowering time. Independently established homozygous lines of the *ddm1* mutant and progeny of independent *METI* antisense transformants showed similar phenotypes (Vongs *et al.*, 1993; Kakutani *et al.*, 1996; Ronemus *et al.*, 1996; Finnegan *et al.*, 1996, 1998a), suggesting that expression of a common group of genes may be dysregulated in these plants.

The mutants generated by decreased DNA methylation differed from loss-of-function mutants because

the severity of the phenotypes increased in successive generations of inbred progeny (Kakutani *et al.*, 1995; Finnegan *et al.*, 1996). A cumulative loss of methylation also occurred at single-copy sequences in *ddm1* homozygous mutant lines followed through several generations (Kakutani *et al.*, 1996), suggesting that this may be associated with the increasing number and severity of developmental abnormalities.

We anticipated that these abnormal phenotypes would be caused by increased or ectopic gene expression as a result of demethylation as has been seen in other cases (reviewed in Finnegan *et al.*, 1998b). Consistent with this, we observed ectopic expression of two floral homeotic genes, *APETALA3* (*AP3*) and *AGAMOUS* (*AG*) in the leaves of *METI* antisense plants that show floral abnormalities resembling *superman* (*sup*) and *sup ag* double mutants (Finnegan *et al.*, 1996). Partial-phenocopy *sup* mutants have been generated by ectopic expression of *AP3* in transgenic plants (Jack *et al.*, 1994), suggesting that the *sup* phenotype of *METI* antisense plants may be due to ectopic expression of *AP3*. However, as part of a general study of unstable mutants with *sup* phenotype, known as *clark kent* (*clk1–7*), Jacobsen and Meyerowitz, (1997) showed that the *sup*-like phenotype in both *clk* and *METI* antisense plants was associated with hypermethylation of the *sup* gene.

Here we report a detailed analysis of changes in methylation at the *SUP* locus in *METI* antisense plants. We found that there is both demethylation and hypermethylation of DNA at this locus. DNA flanking the *sup* gene was demethylated while there was hypermethylation around the transcription start and within the coding region of this gene. There was a correlation between hypermethylation and *sup*-like flowers because antisense families that do not produce *sup*-like flowers showed no hypermethylation within the *SUP* coding region. Hypermethylation of *sup* occurred both in flowers and leaves although *SUP* is normally transcribed only in the developing floral bud (Sakai *et al.*, 1995); we were unable to detect *SUP* transcripts in flowers where *sup* was hypermethylated. The distribution and density of methylcytosine was heterogeneous among different DNA molecules both within a plant and between different plants. A comparison of the pattern of methylation in *METI* antisense and other plants with hypermethylation at *sup* allowed us to conclude that METI is the predominant CpG methyltransferase in *Arabidopsis*, and to determine its sequence specificity.

Materials and methods

Plant growth and analysis

Seeds were sown onto a mix of sand and compost (1:1 mixture) in 20 cm pots; the pots were placed at 4 °C for 48 h before being transferred to controlled environment growth cabinets illuminated with Philips Cool White fluorescent tubes, or metal arc lights at ca. 200 $\mu\xi$, at 22 °C. Short-day photoperiods consisted of alternating cycles of 8 h light/16 h dark; plants grown under continuous light had constant illumination throughout the 24 h cycle. The floral phenotype was scored for the first three flowers on the primary inflorescence and the plants tagged according to phenotype. The floral phenotype was scored again towards the end of flowering. Leaf material or flowers and floral buds were harvested from plants after scoring.

In situ *hybridization*

In situ hybridization experiments for *SUP* and *AP3* were performed as described by Sakai *et al.* (1995).

DNA isolation and Southern hybridization

The protocols for DNA isolation and Southern hybridization were as described by Taylor *et al.* (1989).

Bisulfite treatment of DNA

Two different protocols for bisulfite treatment of DNA were used; both gave essentially identical results (Clark *et al.*, 1994; Jacobsen *et al.*, 2000). The PCR fragments amplified from bisulfite DNA were purified with a QIAquick PCR Purification Kit (Qiagen) and either sequenced directly or, for the analysis of individual DNA molecules, cloned into a plasmid vector before sequencing. Details of the primers used to amplify fragments of the *SUP* gene are available on request. Sequencing was done using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequence reactions were run on an ABI Prism DNA Sequencer Model 377 and analysed with ABI Prism DNA Sequencing Software Version 2.1.2. The GCG sequence analysis package version 8.1 was used for nucleotide comparisons.

Results

METI antisense plants show a variety of abnormal floral phenotypes

Flowers with increased numbers of stamens were observed on two of more than twenty independent transgenic families in which DNA methylation was decreased by a methyltransferase antisense construct. These two families, Nos. 10 and 39, had the greatest decrease in methylation (less than 30% of normal); in the remaining families DNA methylation ranged from 50% to 100% of normal (Finnegan *et al.*, 1996).

Flowers on plants from the T_3 generation of a line, No. 10.5, homozygous for the *METI* antisense locus, showed variable morphology depending on the growth conditions. In short-day photoperiods (8 h light) the phenotype mostly resembled a weak *sup* mutant with a few extra stamens and either a normal gynoecium or a gynoecium in which the carpels were incompletely fused (Figure 1A). In contrast, plants grown in continuous light showed a greater increase in stamen number which was often accompanied by a marked reduction in female reproductive tissue (Figure 1C). In both light conditions, about 25% of plants had normal flowers suggesting that day length may affect the severity of the phenotype, but not the frequency with which plants develop *sup*-like flowers (Table 1).

In the T_4 generation of the same *METI* antisense line, the frequency of plants with wild-type flowers decreased to an average of 5% when grown in either short days or continuous light (Table 1). The flowers were more abnormal than those on T_3 plants grown under the same light regime (Figure 1A–D) and, as with T_3 plants, the floral phenotype was more severe on T_4 plants grown in continuous light than on those grown in short days (Figure 1B and D).

The ovules in *sup*-like flowers on *METI* antisense plants were normal (Figure 1E), unlike *sup* mutants, where ovule morphology is altered (Gaiser *et al.*, 1995). In this aspect the *sup*-like flowers on *METI* antisense plants more closely resemble those on 35S *AP3* transgenic plants where ovule development is unaffected (Figure 1F).

The floral phenotype of plants homozygous for the *sup1-1* mutation was the same when grown in long or short days indicating that the severity of the phenotype in loss-of-function mutants was not affected by photoperiod (not shown).

SUPERMAN *transcripts are not detected in* sup*-like flowers in antisense plants*

To determine whether the *sup*-like flowers on *METI* antisense plants from family 10 were due to ectopic expression of *AP3* or repression of *SUP* we examined the expression pattern of these genes using *in situ* hybridization. In wild-type flower buds, *SUP* hybridized in an adaxial region of whorl 3 adjacent to whorl 4 (Figure 2A; Sakai *et al.*, 1995). There was no hybridization with the *SUP* probe in this region of the *sup*-like floral buds on antisense plants (Figure 2B) indicating that no *SUP* transcript accumulated in these flowers. Consistent with this, *AP3* expression resembled that seen in loss-of-function *sup* mutants (Figure 2C; Sakai *et al.*, 1995). There was no evidence for transcription of *AP3* in the pedicel, in whorl 1 or in the centre of whorl 4 (Figure 2C) indicating that there was no additional ectopic expression of *AP3* in the *sup*-like flowers. Therefore, the *sup*-like phenotype was probably due to the absence of *SUP* transcripts rather than widespread ectopic transcription of *AP3*.

Later in the development of wild-type flowers, *SUP* is expressed in a part of the developing ovule that becomes the funiculus (Sakai *et al.*, 1995). We examined the expression of *SUP* in ovules that developed in residual carpel tissue of flowers on *METI* antisense plants, to determine whether transcription was altered in these tissues. *SUP* was expressed in these ovules, consistent with their normal morphology. In addition, there was some evidence for ectopic expression of *SUP* in the inner carpel wall of these flowers (Figure 2D and E).

Changes in DNA methylation around superman *in* METI *antisense plants*

Southern analyses of DNA cleaved with methylation-sensitive restriction enzymes showed that there are changes in DNA methylation in the region flanking *SUP* in *METI* antisense plants, compared with untransformed C24. The size of *Hpa*II and *Hha*I fragments that hybridized with a 6.7 kb fragment of genomic DNA, which is sufficient to complement *sup* mutations, differed between *METI* antisense family 10 and untransformed C24 plants (Figure 3A, lanes 1 and 2 and lanes 7 and 8). There was demethylation within the recognition sites of these enzymes in DNA from *METI* antisense plants, generating smaller hybridizing fragments than those from control plants. In contrast, digestion with *Eco*RII gave a larger fragment in DNA from the same antisense plants compared with C24

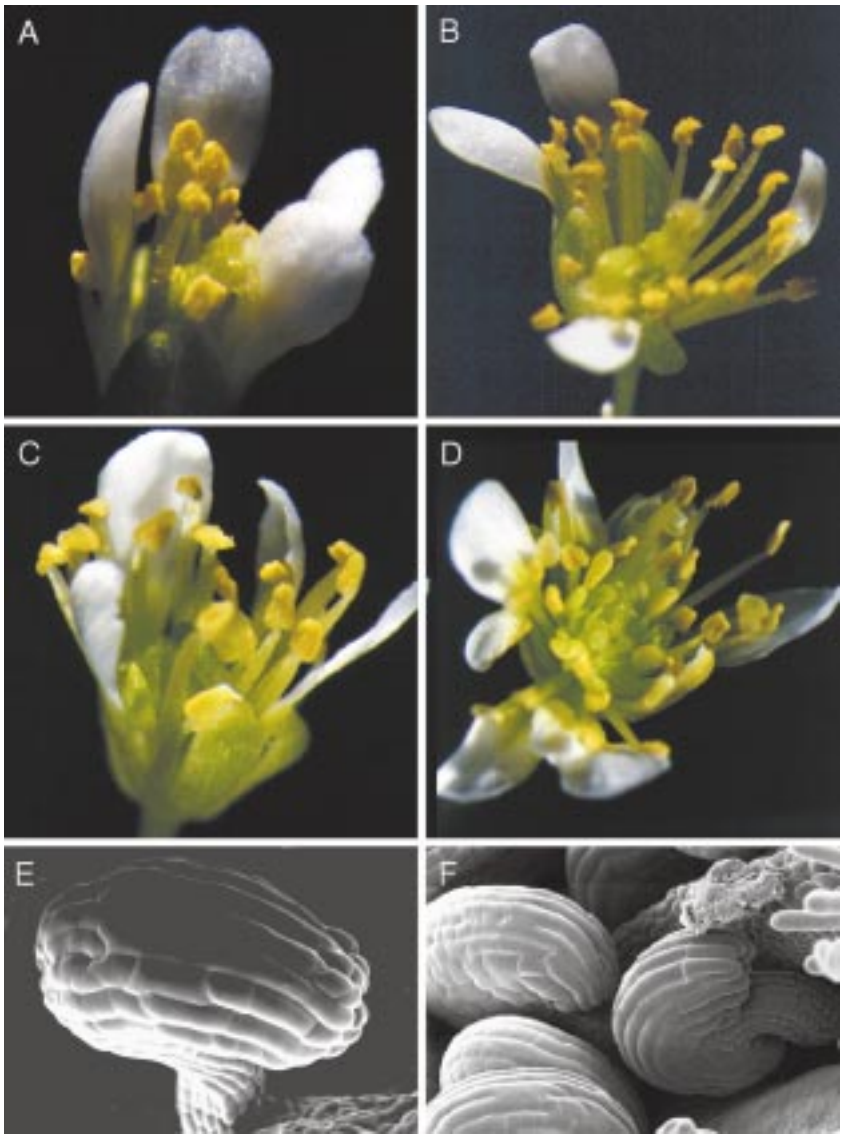


Figure 1. The severity of the *sup* phenotype of flowers on *METI* antisense plants increased in successive generations and when grown in extended photoperiods. Panels A–D show typical examples of flowers with *sup*-like flowers on plants from a homozygous line of antisense family 10. A. T_3 10.5 grown in short days. B. T_4 10.5 grown in short days. C. T_3 10.5 grown in continuous light. D. T_4 10.5 grown in continuous light. Normal ovules developed in the residual gynoecium on a *METI* antisense plants from family 10.5 that had *sup*-like flowers (E) and on transgenic plants with a 35S *AP3* transgene that produced *sup*-like flowers (F).

(Figure 3A, lanes 9 and 10) suggesting that there may be increased methylation of CpA/TpG sequences in the DNA from the antisense plants. Reprobing this blot with probes spanning either the transcribed or 5′-flanking region of the *SUP* gene showed that demethylation of the DNA from antisense plants occurred upstream of the coding region, while the *Eco*RII site that was partially resistant to cleavage was within the transcribed region of the gene. Similar results were obtained with DNA from *METI* antisense family 39 (not shown).

In another *METI* antisense family, 22.6, which does not have *sup*-like flowers, there was partial demethylation of *Hpa*II sites in the 5′-flanking region but no methylation at the *Eco*RII site within the coding sequence (not shown).

In a further study, DNA isolated from plants from family 10 that produced *sup*-like flowers and from un-

Table 1. The frequency of *METI* antisense plants with *sup* flowers was not affected by photoperiod, but increased in successive generations.

Plant line	Photoperiod	^{m}C (total)	Plants with wild-type flowers	Clones without ^{m}C	Average number of ^{m}C in methylated molecules
T3 10.5	8 h (SD)	12.8%	25.0% (102)[a]	12% (25)[b]	11.1
T4 10.5	8 h (SD)	12.0%	5% (145)[a]	ND[c]	ND[c]
T3 10.5	24 h (CL)	13.8%	24.7% (453)[a]	40% (45)[b]	6.6
T4 10.5	24 h (CL)	ND	1.6% (121)[a]	4.5% (22)[b]	9.2

[a]Number of plants scored.
[b]Number of clones sequenced.
[c]ND: not done.

transformed C24 plants was cleaved with *Hin*dIII or *Msp*I, alone or in combination with *Mcr*BC, an endonuclease that specifically cleaves DNA containing at least two methylcytosine residues in the sequence context R^{m}C that are separated by at least 50–80 nucleotides (Sutherland *et al.*, 1992). Digestion of methylated DNA with both enzymes resulted in loss of the fragment generated by *Hin*dIII or *Msp*I alone. Figure 3B shows that *Msp*I sites upstream of the *SUP* locus were incompletely cleaved and therefore were partially methylated in DNA from untransformed plants (lane 3), but were demethylated in *METI* antisense plants (lane 1). The sequence corresponding to the probe DNA, a 1.7 kb *Msp*I fragment located about 4.3–6 kb upstream of the transcription start, was unmethylated in the antisense plants as the *Msp*I fragment was not cleaved by *Mcr*BC (compare lanes 1 and 2). In wild-type plants, this sequence contained methylcytosine towards one end because the fragment resulting from *Msp*I plus *Mcr*BC digestion was smaller than that seen in the corresponding digest of *METI* antisense DNA (lanes 2 and 4).

In contrast, DNA spanning the coding region of *SUP* is not methylated in untransformed plants but is hypermethylated in *METI* antisense plants where a *Hin*dIII fragment from within the coding region was cleaved by *Mcr*BC (Figure 3C, lanes 1–4). Digestion was incomplete indicating that not all DNA molecules contained sufficient methylcytosine to trigger cleavage by *Mcr*BC (lane 2).

These Southern analyses demonstrate that the *sup*-like phenotype in *METI* antisense plants is associated with demethylation of cytosine residues in the region upstream of the *sup* gene and with partial methylation within the coding region of the gene (Figure 3D). To define the location of methylcytosine residues more precisely we analysed all cytosines within a 640 bp region extending from –240 to +400 relative to the start of transcription (Sakai *et al.*, 1995) by bisulfite treatment of DNA followed by PCR amplification and sequencing (Frommer *et al.*, 1992).

The SUPERMAN *gene is methylated in leaves and flowers from* METI *antisense plants*

The Southern analyses utilized DNA isolated from leaves of plants that had been scored for floral phenotype; tissues from plants with *sup*-like flowers were analysed separately from those with normal flowers. As the *SUP* gene is not normally expressed in leaves, we compared the methylation pattern of *SUP* in DNA isolated from leaves with that of flowers taken from the same population of plants. A diagram showing the regions of the *SUP* gene examined by bisulfite sequencing is shown in Figure 4A.

There were no methylcytosine residues within regions 1, 2, 3 or 4 in DNA isolated from either leaves or flowers of untransformed C24 plants. In contrast, there was extensive methylation on both coding and non-coding strands of DNA isolated from leaves or flowers of *METI* antisense plants with *sup*-like flowers (homozygous line 10.5; Figure 4B). The density of methylcytosine residues decreased 5′ to the transcription start which is embedded within a region rich in cytosines that are part of a CT/CA repeat. No methylcytosine was detected more than 35 bp upstream from the transcription start. As the pattern and extent of methylation were very similar in both leaves and flowers, all subsequent analyses were done on DNA isolated from leaves of plants for which the floral phenotype had been determined.

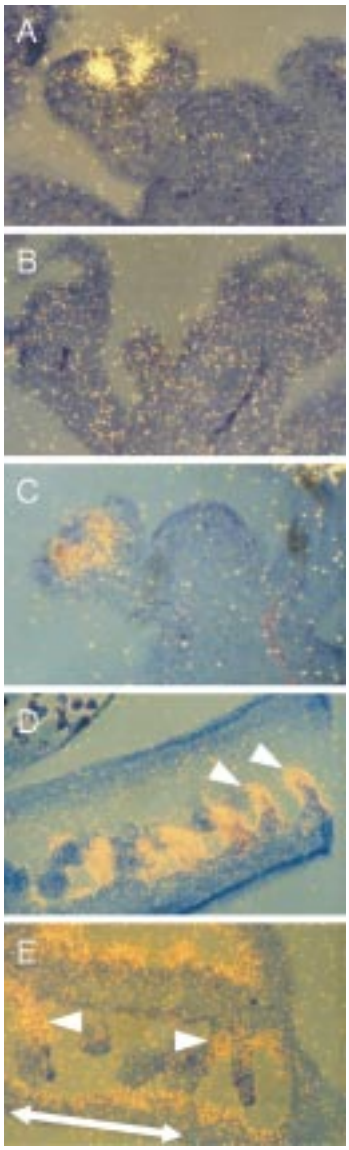


Figure 2. There was no detectable *SUP* transcript in the floral bud, combined with ectopic expression in the remaining ovaries of *sup* flowers on *METI* antisense plants. A. Wild-type flower bud hybridized with *SUP* probe. B. Bud at about the same stage of development from *METI* antisense plant with *sup* flowers hybridized with *SUP* probe. C. Bud from *METI* antisense plant with *sup* flowers hybridized with *AP3* probe. D. *SUP* expression in gynoecium from an untransformed C24 plant; arrowheads indicate *SUP* expression developing ovules. E. *SUP* expression in gynoecium from a *METI* antisense plant with *sup* flowers; arrowheads indicate *SUP* expression in developing ovules, while the double-headed arrow indicates ectopic expression in the carpel wall.

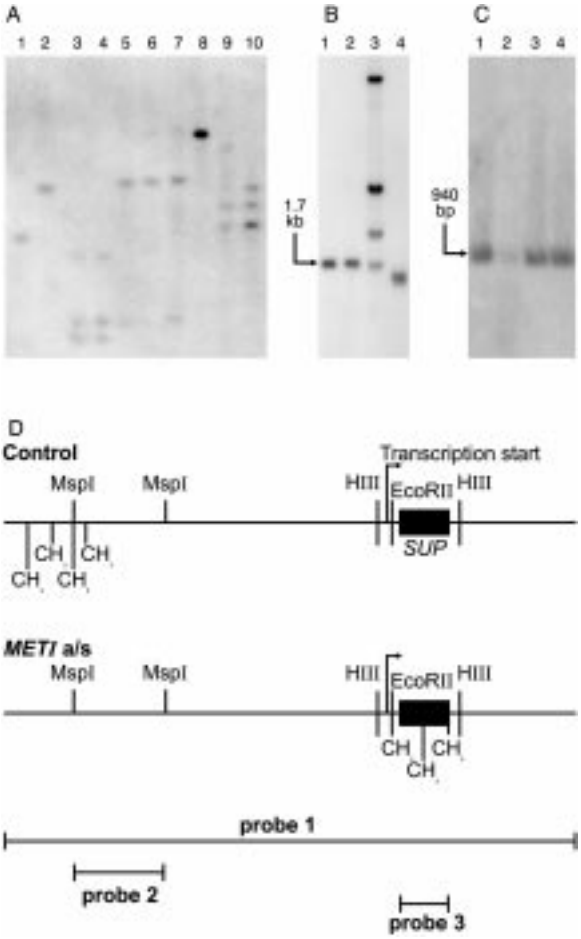


Figure 3. Southern hybridization showing changes in methylation around that *SUP* locus. A. DNA from C24 (lanes 2, 4, 6, 8, 10) and *METI* antisense plants from family 10.5 (lanes 1, 3, 5, 7, 9), which have *sup* flowers, was digested with *Hha*I (lanes 1, 2), *Hae*III (lanes 3, 4), *Msp*I (lanes 5, 6), *Hpa*II (lanes 7, 8) or *Eco*RII (lanes 9, 10) and hybridized with probe 1, a 6.7 kb probe spanning the *SUP* coding region and sequences 5' to the gene (see D). B. DNA from C24 (lanes 3, 4) and from *METI* antisense plants from family 10.5 (lanes 1, 2), which have *sup* flowers, was digested with *Msp*I (lanes 1, 3) or *Msp*I and *Mcr*BC (lanes 2, 4) and hybridized with probe 2, an *Msp*I fragment located about 4.3–6 kb upstream of the *SUP* coding region (see D). C. DNA from C24 (lanes 3, 4) and from *METI* antisense plants from family 10.5 (lanes 1, 2), which have *sup* flowers, was digested with *Hin*dIII (lanes 1, 3) or *Hin*dIII and *Mcr*BC (lanes 2, 4) and hybridized with probe 3, a *Hind*III fragment isolated from the coding region of *SUP* (see D). D. Schematic diagram showing the location of the probes used and the location of methylcytosine as determined by Southern hybridization. The *Eco*RII and *Hpa*II (shown here as *Msp*I) sites whose methylation status changes giving rise to altered patterns of hybridization in Southern analyses are shown. The location of the *Hha*I site which is demethylated in *METI* antisense plants has not been determined.

Methylation at SUPERMAN *is correlated with the* sup-*like phenotype*

We examined the methylation pattern within these same regions of DNA isolated from other antisense families that do or do not have *sup*-like flowers to determine whether methylation at *SUP* is strictly correlated with the development of *sup*-like flowers. DNA from family 39, which had *sup*-like flowers, showed the same pattern of methylation as seen in plants from family 10.5 with *sup*-like flowers (Figure 4B).

No *sup*-like flowers were seen on plants from family 22.6; there was also no evidence of methylcytosine around the start of transcription or within the coding region of *SUP* in these plants. Plants of family 10.5, which have normal flowers, had little, if any, methylcytosine in the *SUP* gene, as judged by sequencing the bulk PCR fragments. Sequencing of individual clones from this pool showed that most (44/48) had no methylcytosine while the remaining clones had only 1–5 methylcytosine residues. These data support the observation that methylation around the transcription start and within the coding sequence is associated with the development of *sup*-like mutant flowers, and conversely that this gene is not methylated in plants with normal flowers.

Methylation at SUPERMAN *is heterogeneous*

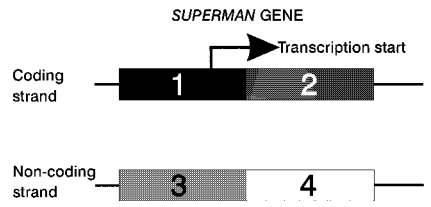
The pattern of methylation of individual DNA molecules was examined by sequencing cloned PCR products that were amplified from bisulfite-treated DNA, isolated from leaves from a population of *METI* antisense plants with *sup*-like flowers. The distribution and density of methylcytosine varied considerably between different molecules of DNA (Figure 4c). Some DNA molecules were densely methylated with up to 30% of cytosines methylated, but other DNA molecules from the same preparation of DNA contained no methylcytosine in the region sequenced.

When DNA from individual plants was examined, a similar pattern of heterogeneous methylation including molecules that lacked methylation was observed, suggesting that heterogeneity of methylcytosine density occurs within a plant (Figure 4e).

Target specificity of methyltransferase(s) that catalyse SUPERMAN *hypermethylation*

The most frequently methylated cytosines at the *sup* locus in *METI* antisense plants are those within symmetric triplets CpApG and CpTpG. Asymmetric

A



B

Plant line	Floral Phenotype	Region 1	2	3	4	Upstream demethyl^n
C24 (control)	wildtype	–	–	–	–	No
METI antisense #22.6	wildtype	–	–	–	–	Partial
METI antisense #39	*sup*	+	+	+	+	Yes
METI antisense #10	*sup*	+	+	+	+	Yes

+ methylated - not methylated

Figure 4. The distribution and density of methylcytosine was determined by genomic sequencing of bisulfite-treated DNA from *METI* antisense plants. A. Four regions spanning 640 bp on both the coding and non-coding strands were examined by sequencing bisulfite-treated DNA. Region 1 contains the transcription start site embedded within a CT/CA repeat; the start of translation lies near the beginning of region 2. B. The coding region of the *SUP* gene was methylated in *METI* antisense families that had *sup*-like flowers, but remained unmethylated in an antisense family that never had *sup*-like flowers. In C24 plants, DNA at least 4 kb upstream of *SUP* was methylated; this region was unmethylated in *METI* antisense plants (upstream demethylation). C. Location of methylcytosine in independent cloned PCR amplimers for plants from the T_3 generation of homozygous line 10.5. The data shown here are from Region 1 (see A, 370 bp) covering the start of transcription which is indicated with an arrow. Each horizontal line represents an individual PCR fragment and the vertical lines indicate the relative position of cytosines within this region. The positions of the symmetrically located cytosines are indicated by the sequence given above the grid. Dots on the grid indicate the position of methylcytosine for each PCR fragment and the number of methylcytosines in each sequenced fragment is indicated to the left of the line representing that clone. The data compiled from all the sequenced clones is shown below the grid; each dot indicates that methylcytosine occurred at this location in one PCR fragment. d. Location of methylcytosine in independent cloned PCR amplimers from Region 1 from a population of plants from the T_4 generation of homozygous line 10.5. (See legend to C for explanatory notes.) e. Location of methylcytosine in cloned PCR amplimers from Region 4 (270 bp, see A) from one T_4 plant from homozygous line 10.5. (See legend to C for explanatory notes.)

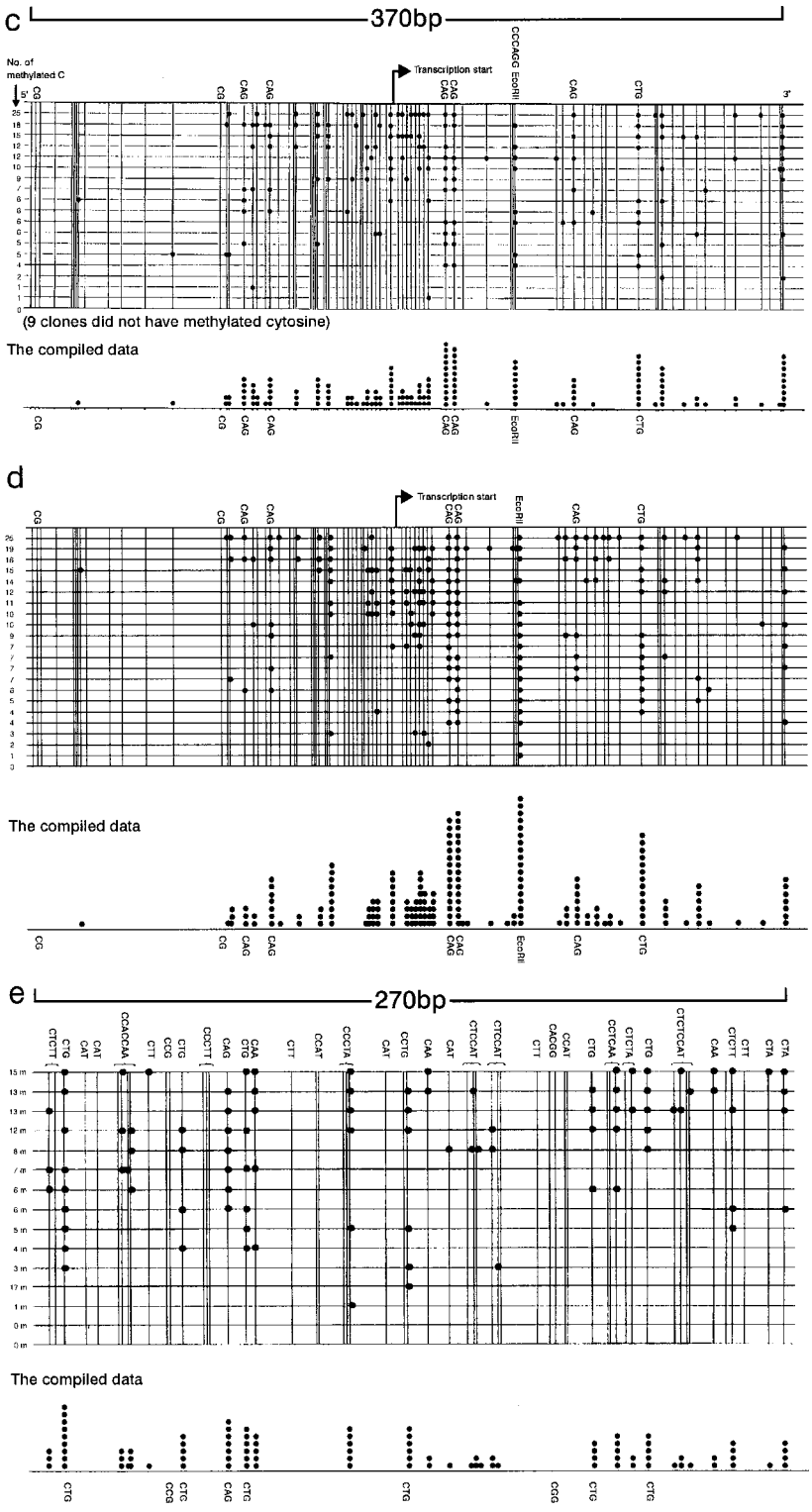


Figure 4. Continued.

Table 2. Site specificity of methylation at the *superman* locus in *METI* antisense plants. The location of regions 1 and 4 is indicated on Figure 4A.

DNA triplet	Region 1[a]		Region 4[b]	
	number of times a triplet occurs	% mC at triplet	number of times a triplet occurs	% mC at triplet
CpApA	8	19	5	30.5
CpApC	4	1.7	2	7.5
CpApG	6	52	1	57.5
CpApT	9	2.7	9	2.8
CpCpA	6	2.8	7	3.9
CpCpC	4	1.3	2	0
CpCpG	–		1	0
CpCpT	2	0.8	4	6.3
CpGpA	–		1	0
CpGpC	–		–	
CpGpG	–		1	5
CpGpT	1	0	–	
CpTpA	7	19	4	35.6
CpTpC	17	14.9	8	8.4
CpTpG	1	66.7	6	45.4
CpTpT	10	4.8	7	12.5

[a]Number of clones with methylcytosine = 62.
[b]Number of clones with methylcytosine = 40.

sequences were also methylated, with cytosines in CpApA and CpTpA being more commonly methylated than those in other contexts (Table 2); cytosines least frequently methylated were those in the triplets CpCpN and CpGpN. The pattern of methylation is similar to that reported for the *sup* locus in *clk* mutants (Jacobsen and Meyerowitz, 1997), with one exception: CpG was methylated at high frequency in *clk* mutants but was rarely methylated in *METI* antisense DNA.

To determine whether the difference in methylation pattern in *clk* vs. *METI* antisense plants was due to genetic differences between ecotypes C24 (*METI* antisense) and L*er* (*clk*), we compared the pattern of hypermethylation in mutants *clk3* (L*er*) and *metI* (also known as *ddm2*) introgressed three times into L*er* from Col (E. J. Richards, personal communication). We sequenced cloned PCR fragments amplified from bisulfite-treated DNA from each mutant. We found that the density of methylcytosine was higher in DNA from the *clk3* mutant (average 56 ^{m}C/clone) than in the *metI* mutant (average 19 ^{m}C/clone); in both lines, most methylcytosine residues were in asymmetric sites (Table 3). The lower overall frequency of methylcytosine seen in the *metI* mutant is comparable with that seen in *METI* antisense plants (Table 1). In *metI* plants the frequency of ^{m}CpG was very low (average 0.6 ^{m}CpG/clone) with most clones sequenced having only one or no methylcytosines in this context. The occurrence of ^{m}CpG was higher in the *clk3* mutant (average 4 ^{m}C/clone), with all clones having between two and six methylated CpG sites (Table 3). In contrast, the frequency of methylcytosine in CpNpG triplets was comparable for the two mutant lines (9 ^{m}CpNpG in *clk3* versus 8 ^{m}CpNpG in *metI*). These data suggest that METI is the predominant enzyme catalysing CpG methylation and that loss of METI activity directly or indirectly decreases the frequency of methylation at asymmetric cytosine.

We have shown previously that antisense-null progeny, which did not inherit the *METI* antisense transgene from their hemizygous parent, inherited the low methylation and abnormal phenotypes observed in the parent (Finnegan *et al.*, 1996). We examined the pattern of methylation at *sup* in antisense-null plants with *sup*-like flowers to determine whether METI contributed to hypermethylation at *sup*. Direct sequencing of the PCR products showed that in these antisense-null plants, the density of methylcytosine was similar to that in sibling plants which inherited the methyltransferase antisense but that, in antisense-null plants, methylcytosine residues occurred at CpG dinucleotides; these residues were almost uniformly

Table 3. The frequency of methylcytosine in CpG dinucleotides at the *sup* locus is reduced in *metI* mutant plants. The sequenced region covered 705 bp of the coding strand which includes 166 cytosines nine of which are located in CpG dinucleotides.

Clone	*clk3*			*metI*		
	number of ^{m}C	number of ^{m}CpG	number of $^{m}CpNpG$	number of ^{m}C	number of ^{m}CpG	number of $^{m}CpNpG$
1	32	2	9	11	2	5
2	47	3	9	26	1	9
3	30	5	4	12	0	8
4	20	4	5	11	1	5
5	79	4	11	24	0	8
6	110	6	11	19	0	10
7	40	4	10	17	0	9
8	86	3	11	30	1	8
Mean	55.5	3.9	8.8	18.8	0.6	7.8
SE	11.4	0.4	1.0	2.6	0.3	0.6

unmethylated in plants that had the *METI* antisense (not shown). Therefore, the enzyme(s) that catalyses hypermethylation at *sup* cannot substitute for METI, but rather methylates sites not normally methylated by the METI protein. These observations strongly suggest that differences in methylation pattern between *clk* and *metI* mutant or *METI* antisense plants are due solely to the absence of METI in the latter and that, when present, METI contributes to hypermethylation of *sup*.

The severity of the sup*-like phenotype does not correlate with methylation density at* superman

The severity of the floral phenotype, including the *sup*-like phenotype, was enhanced in successive generations of *METI* antisense plants from family 10 and when plants were grown under continuous light compared to short days (Figure 1). The methylation patterns for T_3 and T_4 plants, grown under continuous light, were similar (Figure 4c and d). Day length did not affect the pattern or density of methylation at *sup* in DNA isolated from plants of either generation. Similarly, the global level of cytosine methylation was invariant between generations and growth conditions (Table 1).

The enhancement of the *sup*-like phenotype in the antisense plants, by increased day length and in successive generations, was probably due to altered expression of other genes; for example, we have observed hypermethylation and loss of *AG* transcripts in plants with *sup ag* flowers (Jacobsen *et al.*, 2000). This idea is supported by the observations that mutation at *ag* enhances the *sup* mutant phenotype (Bowman *et al.*, 1992) and the *clk* phenotype is more severe in *ag/AG* heterozygotes (Jacobsen, unpublished).

Discussion

The *sup*-like phenotype observed in two independent *METI* antisense families is associated with hypomethylation of DNA at least 4 kb upstream of the coding region of *sup* and with hypermethylation of DNA around the transcription start and within the coding region. In wild-type flowers, *SUP* is transcribed in whorl 3 adjacent to whorl 4 in the developing floral bud (Sakai *et al.*, 1995); hypermethylation of *sup* correlated with the loss of detectable transcripts in floral buds. It is likely that the molecular basis for the *sup*-like phenotype in *METI* antisense and *clk* mutants is the same, as the latter also showed an absence of *SUP* transcripts by *in situ* hybridization and similar changes in methylation status of the gene (Jacobsen and Meyerowitz, 1997).

A second domain of *SUP* expression occurs on the inner surface of the carpels and, later, in the funiculus of developing ovules (Sakai *et al.*, 1995), where it mediates asymmetric cell division of the outer integument of the ovule (Gaiser *et al.*, 1995). In contrast to loss-of-function *sup* mutants, normal ovules developed in residual carpel tissue of the *sup*-like flowers on *METI* antisense plants. Consistent with this, *in situ* hybridization with a *SUP* probe showed that this

gene was expressed in the developing ovules in *METI* antisense-induced *sup*-like flowers. This suggests that the *SUP* promoter may have separate domains regulating transcription in the developing flower and ovules, which are differentially regulated in the flowers of *METI* antisense plants. Perhaps hypermethylation of *SUP* does not prevent expression in the ovule or the gene may not be hypermethylated in this tissue.

Our data suggest that hypermethylation may not be the primary event repressing transcription of *sup*. In *METI* antisense plants that had *sup*-like flowers, the pattern of methylation at *sup* was heterogeneous among individual DNA molecules isolated from leaves from a single plant. While some molecules contained up to 30% methylated cytosines, other molecules from the same plant had no methylated cytosine residues in the region examined. Consistent with this, Southern analyses demonstrated that although the majority of DNA at the *sup* locus was hypermethylated, some molecules were unmethylated (Figure 3c). As DNA was isolated from plants that had *sup*-like flowers this suggests that something else, possibly condensation of chromatin around *sup*, is the primary event leading to transcriptional repression. Methylation of *sup* DNA may be a secondary event that stabilizes the transcriptionally inactive state of the gene. The primary event controlling expression of the *Pl-Blotched* gene of maize, a gene controlling anthocyanin pigmentation, also appears to be condensation of chromatin which is subsequently stabilized by methylation (Hoekenga *et al.*, 2000).

The *SUP* locus of *Arabidopsis* appears to be particularly susceptible to hypermethylation after events that perturb the genome, for example, the genome-wide demethylation that occurs in *METI* antisense plants or *ddm1* mutants (Vongs *et al.*, 1993; Jacobsen *et al.*, 2000). Like *clk1–7*, the allelic mutations *floral organ number* (*fon*) and *carpel* (*car*), which also show hypermethylation at *sup*, were identified in plants that had been exposed to various mutagenic agents (Jacobsen and Meyerowitz, 1997; Hui and Ma, 1998; Rohde *et al.*, 1999), suggesting that mutagenesis can perturb DNA methylation. It has been proposed that hypermethylation at *sup* in the *clk*, *fon1–3* and *car* mutants is associated with a single base change of G to A at base +466 relative to the translation start (Rohde *et al.*, 1999). We have sequenced this region in C24 and *METI* antisense plants and found that this base is a G in both wild-type and antisense plants; conversely, the L*er* accession in our collection and the *clk* mutants have an A in this position. It is unlikely, therefore, that the base at +466 influences the occurrence of hypermethylation of the surrounding cytosines. Perhaps the common event that triggers hypermethylation of *sup* in mutagenized and *METI* antisense plants is an alteration in chromatin structure resulting either from demethylation of DNA flanking *SUP* or from DNA repair in mutagenized plants. Hypermethylation at *sup* may result from the activity of a plant defence mechanism that detects changes in chromatin as part of a genome management strategy to control transposable elements and invading DNA. Activation of a plant defence system may account for the paradox where regions of hypermethylated DNA occur in *METI* antisense plants with genome-wide hypomethylation.

The *AGAMOUS* gene is also the target of hypermethylation and inactivation in some *METI* antisense plants (Jacobsen *et al.*, 2000). There is a gradient of hypermethylation as the *AG* gene is inactivated and methylated in about 10% of plants showing the *sup* phenotype and never in plants with *SUP* flowers (Finnegan, unpublished). Other abnormal floral phenotypes are observed on *METI* antisense plants; these may also be due to hypermethylation and inactivation of the corresponding genes, for example, *LEAFY* and *APETALA1*, but the methylation status of these genes has not been analysed in detail.

Little is known about the methyltransferase(s) that catalyse hypermethylation at *sup*, transposable elements or transgenes. Our data demonstrate that METI is the predominant enzyme catalysing methylation of cytosines in CpG dinucleotides; METI can contribute to hypermethylation at *sup* but it is not essential for either establishing or maintaining hypermethylation of this locus. Loss of METI activity also resulted in a lower frequency of asymmetric methylation at the *sup* locus in *metI* compared with *clk3*, suggesting that METI is involved, directly or indirectly, in methylation of asymmetric sites. In contrast, METI is not important for the methylation at CpNpG sequences as the frequency of methylation at these sites was the same in *clk3* and *metI* plants.

Asymmetric methylation may be catalysed by METI directly, or by another methyltransferase that is attracted to DNA containing ^{m}CpG which may provide a core from which asymmetric methylation spreads. However, methylation at asymmetric cytosine residues occurred in some DNA molecules that contained no symmetrically methylated cytosines, suggesting that other factors can stimulate *de novo* methylation of asymmetric cytosines. *Arabidopsis* plants

have a number of putative methyltransferases that could be important for *sup* hypermethylation (Genger *et al.*, 1999). It is reasonable to suppose that an enzyme with *de novo* methylation activity is required, the most likely candidate being DRM2, an *Arabidopsis* homologue of the mouse Dnmt3 *de novo* methyltransferase (Okano *et al.*, 1998; Cao *et al.*, 2000). Analysis of the pattern of methylation at *sup* in the presence of antisense constructs directed against these other methyltransferases may provide insight into which enzymes are involved.

While *sup* hypermethylation mutants have been generated in the laboratory upon mutagenesis or in plants with reduced DNA methylation (Jacobsen *et al.*, 2000), epimutations also occur in wild populations. Hypermethylation of a gene controlling floral symmetry has recently been reported in a naturally occurring mutant of *Linaria vulgaris* (Cubas *et al.*, 1999). As reported here, hypermethylation was associated with transcriptional repression of the *LCYC* gene resulting in loss of floral asymmetry; the molecular events that triggered abnormal methylation in this mutant, which was originally described more than 250 years ago, are unknown. Epimutations, such as those at *SUP* and *LCYC*, may be more common than previously thought and may arise in response to other events that perturb the DNA methylation machinery which is involved in genome management.

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