Chapter Three

The effect of reduced DNA methylation on the flowering time and vernalization response of *Arabidopsis thaliana*

3.1 Introduction

The time at which a plant flowers is influenced by environmental conditions, including light intensity, photoperiod, and temperature. The flowering of plants which grow in temperate climates or high altitudes can be promoted by the prolonged low temperatures experienced in winter, ensuring that flowering and seed set will take place in the favourable conditions of spring and summer. This promotion of flowering after prolonged cold temperatures is termed vernalization.

For a plant to be vernalized, the shoot apex, or cells that give rise to a shoot apex, must be directly exposed to low temperatures (Purvis, 1940; Schwabe, 1954). If other plant parts, but not the shoot apex, are exposed to low temperatures, flowering will not be promoted, demonstrating that there is no tissue-transmissible vernalization signal (Metzger, 1988a). Mitotic activity is also required for promotion of flowering. Plants which flowered were regenerated from leaves of *Lunaria biennis* containing cells mitotically active during the cold treatment, while plants regenerated from leaves which were mitotically inactive did not flower (Wellensiek, 1964). In *Thlaspi arvense* and *Arabidopsis thaliana*, plants regenerated from leaf petioles or root tips which contained dividing cells during cold treatment flowered earlier than plants regenerated from untreated tissues (Metzger,

1988a; Burn *et al*., 1993a; J.D. Metzger, personal communication). This demonstrates that the vernalization signal is transmitted stably through mitosis. The signal is not transmitted through meiosis; progeny of vernalized plants require cold treatment to flower early (Lang, 1965).

These characteristics of vernalization are consistent with the vernalization signal being transmitted by a modification of DNA such as DNA methylation, as suggested by Burn *et al*. (1993a). Methylation patterns are stably transmitted through mitosis (Razin and Riggs, 1980; Wigler *et al*., 1981; Stein *et al*., 1982), and remethylation of newly synthesized DNA occurs immediately after DNA replication (Billen, 1968; Leonhardt *et al*., 1992). The linkage of remethylation and DNA replication is consistent with the requirement for cell division for effective vernalization. If cold temperatures cause alterations in DNA methylation patterns, these changes could be made during cold treatment immediately following DNA replication in dividing cells. Correlative evidence also links methylation and vernalization. Cold treatment reduces methylation levels in cell cultures of *Nicotiana plumbaginifolia* and Arabidopsis whole plants (Burn *et al*., 1993a; Finnegan *et al*., 1998b). Methylation levels are restored when plants are returned to normal growth temperatures (Finnegan *et* al., 1998b). Application of 5-aza-C, a demethylating agent (Jones, 1985) causes early flowering in the vernalization responsive crucifer *Thlaspi arvense*, vernalization responsive ecotypes and late flowering mutants of *Arabidopsis thaliana* (Burn *et al*., 1993a), and winter wheat (Brock and Davidson, 1994). Spring wheat and late flowering mutants of Arabidopsis which do not respond to vernalization do not flower early in response to 5-aza-C (Burn *et al*., 1993a; Brock and

Davidson, 1994). The specificity of the response strengthens the case for the involvement of cytosine methylation in vernalization.

Burn *et al*. (1993a) hypothesized that methylation of specific DNA sequences prevents flowering. Such sequences would be expected to occur in the promoter region of a gene(s) critical for flower initiation, and to block flowering by preventing transcription of this gene(s). Treatment with cold or 5-aza-C would remove this methylation block, leading to the promotion of flowering.

As well as inhibiting methylation, 5-aza-C is a general inhibitor of transcription (Cedar, 1988). Although the early flowering response to 5-aza-C was limited to plants that also showed a vernalization response, the possibility was raised that the early flowering response to 5-aza-C was not caused by its demethylating effects. To test whether decreasing DNA methylation by genetic rather than chemical methods would cause early flowering, two classes of Arabidopsis plants with reduced methylation levels were used. The first class contained an antisense construct of the Arabidopsis methyltransferase gene *METI* (Finnegan and Dennis, 1993; Finnegan *et al*., 1996), and the second class were mutants at the *DDM1* (decreased DNA methylation) locus (Vongs *et al*., 1993; Kakutani *et al.*, 1995).

An antisense construct of the Arabidopsis methyltransferase gene *METI*, the first member of a small family of Arabidopsis methyltransferase genes to be cloned (Finnegan and Dennis, 1993) was made using a 2.8 kb fragment of the *METI* cDNA that encodes the

putative methyltransferase domain (520 aa) and ~330 aa of the amino terminal domain (Finnegan *et al*., 1996). This construct, under the control of a constitutive promoter, was transformed into Arabidopsis plants of ecotype C24 (Finnegan *et al*., 1996), an Arabidopsis ecotype which flowers later than the commonly used ecotypes Columbia (Col) and Landsberg *erecta* (L*er*) (Sanda and Amasino, 1995). The progeny of four transgenic plants, designated #10, #4, #22.6 and #39, were found to have lowered methylation levels relative to wildtype. Hypomethylation was detected in both centromeric and ribosomal repeat DNA, and in CG dinucleotides within *Taq*I sites.

METI antisense plants with reduced methylation showed a pleiotropic phenotype including small stature, reduced apical dominance, reduced fertility, altered leaf morphology, and homeotic transformations of floral organs. The phenotype was variable, with different plants showing subsets of these phenotypic effects. Floral abnormalities were most severe in families #10 and #39, which had the lowest levels of methylation. In family #10, floral abnormalities worsened through four successive generations. Plants from T2, T3 and T4 generations of four families showing demethylation, #4, #10, #22.6, and #39 were used in the studies of flowering time reported here.

Decreased DNA methylation mutants (*ddm*) were identified from an ethyl methane sulfonate mutagenized population of Arabidopsis ecotype Columbia (Vongs *et al*., 1993). Cytosine methylation levels were reduced to 25-30% of wildtype levels in plants homozygous for mutations at the *DDM1* allele (Vongs *et al*., 1993). Although the *ddm1* mutation is recessive, methylation levels in heterozygotes produced by outcrossing to

wildtype Columbia were intermediate to those of the parents. For each successive generation of plants backcrossed to Columbia, methylation levels increased by increments of approximately 50% of the difference in methylation level of the parents. This is consistent with the increases in methylation being due to loss of hypomethylated DNA segments by segregation, and suggests that *de novo* methylation occurs at a very low rate (Vongs *et al*., 1993). DNA methyltransferase activity in *ddm1* mutants was no lower than in wildtype Columbia, and levels of S-adenosylmethionine, the methyl group donor, were not altered in the mutant (Kakutani *et al*., 1995). The predicted DDM1 gene product has homology to the *Drosophila* SNF/SWI group of proteins (Jeddeloh *et al*., 1999), which are involved in chromatin modelling (reviewed in Gregory and Hörz, 1998; Varga-Weisz and Becker, 1998).

When first identified, homozygous *ddm1* mutants displayed only slight morphological changes (Kakutani *et al*., 1995), but plants generated by repeated self pollination showed severe developmental abnormalities including reduced or increased apical dominance, reduced stature, late flowering, small leaves, increased cauline leaf number, reduced fertility, and, in some cases, abnormal flowers (Kakutani *et al*., 1996). Like the *METI* antisense transgenic line (Finnegan *et al*., 1996), individual plants among the *ddm1* homozygotes exhibited different subsets of the spectrum of phenotypes. The inheritance of two commonly seen combinations of phenotypes was examined; both were found to be caused by heritable lesions at single Mendelian loci, unlinked to the *ddm1* locus (Kakutani *et al*., 1996). Paralleling the emergence of abnormal phenotypes, loss of methylation was observed in single copy sequences of plants generated by repeated self

pollination (Kakutani *et al*., 1996).

The *ddm1* homozygotes used in the observations of flowering time reported here were from an early generation resulting from self pollination, and these plants did not exhibit severe developmental abnormalities.

3.2 Materials and Methods

3.2.1 Plant lines

Arabidopsis plants of ecotype C24 were transformed with an antisense construct to a region of the Arabidopsis methyltransferase gene *METI* by Finnegan *et al.* (1996). Lines from transgenic families #10, #4, #22-6 and #39 were used. Family #10 has three copies of the antisense transgene inserted at a single locus. T2 plants 10.4 and 10.5 were homozygous for this locus, while T2 plant 10.1 was hemizygous. The T3 progeny of these plants were used; the progeny of plant 10.1 was segregating for the presence of the antisense transgene (Finnegan *et al.*, 1998b). The T4 progeny of two T3 plants 10.1.4 and 10.1.8 were also used; T3 plant 10.1.4 was hemizygous for the antisense transgene, while T3 plant 10.1.8 lacked the antisense transgene (Finnegan *et al*., 1998b; E.J. Finnegan, personal communication). Family #4 has three copies of the antisense transgene inserted at a single locus**.** No segregation data is available for this family (Finnegan *et al*., 1996; E.J. Finnegan, pers. comm.). Family #22-6 has four copies of the antisense transgene, three of which are inserted at a single locus while the fourth is

unlinked. T2 plant 22-6.9 was homozygous for the 3 linked copies and hemizygous for the unlinked copy, while T2 plant 22-6.11 was homozygous for all four copies (Finnegan *et al*., 1998b). The T3 progeny of these plants were used. Family #39 has five copies of the transgene; the T3 progeny of T2 plant 39.35 were used, but due to the family's very low fertility, no segregation data is available (Finnegan *et al*., 1998b).

3.2.2 Assay for the presence of the transgene

The antisense construct contains the selectable *Npt*II gene (Finnegan *et al*., 1996), allowing the presence of the transgene to be detected by a polymerase chain reaction, using template DNA prepared from a single leaf (Klimyuk *et al*., 1993). This DNA extraction procedure and the conditions for *Npt*II PCR amplification and analysis are described in Sections 2.2.4 and 2.3.2.

3.2.3 Measurement of flowering time

Seed sterilization and growth conditions for the flowering time experiments are described in Section 2.1. The lines, growth conditions and lighting used for each experiment are shown in Table 3.1. For most lines, 40 plants were grown for both the control and the vernalization treatment; in some cases, only 20 plants were grown. The photoperiod was 8 hours or 16 hours as indicated in Table 3.1, and fluorescent tubes were used in all but one experiment, in which metal arc lights were used. Light intensity varied between experiments as indicated, and declined towards the sides of the cabinet. As described in

Table 3.1: Plant lines and growth conditions for experiments to measure flowering time.

Light intensities are given in μEinsteins (μE; μM m^{-2} s^{-1}).

Experiment	Lines used	Photoperiod	Light type	Light intensity
1	C24 T2#10 T3#10.1 T3#10.4	8 hours	Phillips TLMF 140W/33RD	not measured
2	C24 T2#4 T3#4.2 T3#22.6.11	8 hours	Phillips TLMF 140W/33RD	not measured
3	C24 T3#4.2 T3#22.6.11 T3#10.5 Columbia *ddm*	8 hours	Sylvania GTE F58W/133	160-190 μE
4	C24 T3#39.35 T3#10.1 T4#10.1.8 T3#10.5 Columbia *ddm*	8 hours	Osram 58W/21-84O	140-180 μE
5	C24 T3#39.35 T3#10.1 T4#10.1.4 T4#10.1.8 T3#22.6.9 T3#22.6.11 Columbia *ddm*	8 hours	Osram 58W/21-84O	130-170 μE
6	C24 T3#4.2 T3#22.6.11 T3#10.5	16 hours	Phillips TLMF 140W/33RD	80-100 μE
7	C24 T3#4.2 T3#22.6.11 T3#10.5 Columbia *ddm*	16 hours	Phillips TLMF 140W/33RD	180-200 μE

Section 2.1, plants were grown in glass tubes. These tubes were placed in racks of twenty, which were moved within the cabinet daily to ensure that all plants received equal illumination. The plants were observed daily, and the dates of germination, defined as cotyledon emergence, and bolting, defined as first elongation of the primary inflorescence, were recorded, as was the rosette leaf number at the time of bolting. The Mann-Whitney *U* test was used to determine the statistical significance of observed differences in flowering time (Snedecor and Cochran, 1967).

Initial experiments used Phillips TLMF 140W/33RD fluorescent tubes. When these were no longer available, Osram 58W/21-84O and Sylvania F58W/133 tubes were used. As Figure 3.1 shows, the spectra from the Phillips and Sylvania tubes were almost identical, while the spectrum from the Osram tubes differs considerably (David Bagnall, personal communication). Flowering was delayed when Osram tubes were used.

3.2.4 Estimation of DNA methylation

Methylation of cytosines in *Taq*I sites was measured by a thin layer chromatography method, described in detail in Section 2.4. *Taq*I recognizes the sequence TCGA, and cleaves 5' to the cytosine residue regardless of its methylation status. Average fragment size after cleavage with *Taq*I is less than 500 bp (Finnegan *et al*., 1996). Measurements were made on DNA from both bulked plants and individual plants. DNA was isolated from bulked plants of each transgenic line using the CTAB-CsCl gradient method described in Section 2.2.1. DNA from individual plants of lines T3#10.1 and T3#10.4

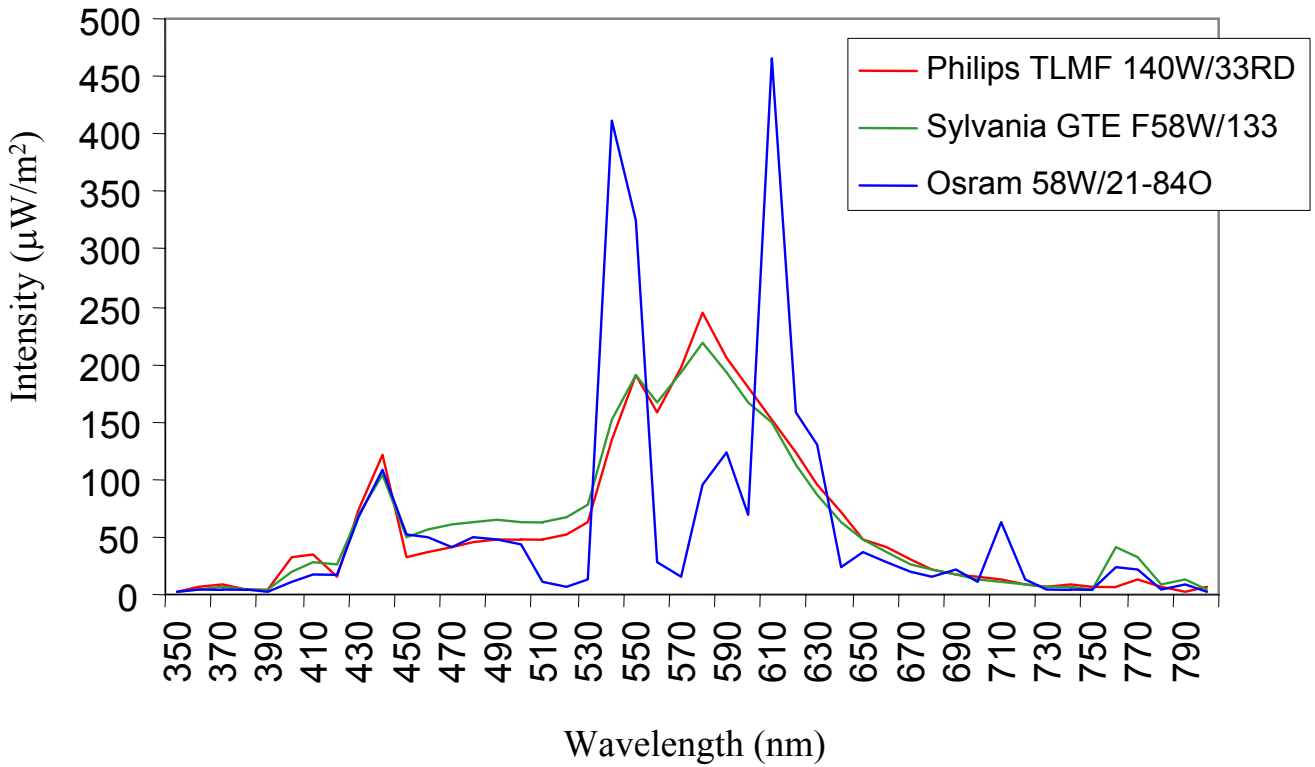


Figure 3.1: Spectra of three fluorescent light sources used in flowering time experiments. Spectra were measured at equivalent light intensities of 150 µmol/m^2/s. These data were provided by David Bagnall, CSIRO Plant Industry.

was isolated using a plant DNA mini-preparation method (modified from Dellaporta *et al*., 1983) described in Section 2.2.2.

3.3 Results

3.3.1 Plants with decreased DNA methylation flower early

Initial experiments to test the effect of *METI* antisense-mediated demethylation on flowering time in ecotype C24 were done in short day conditions (8 hours light, 16 hours dark) to maximise the difference in flowering time between unvernalized and vernalized plants. This increased the opportunity of observing any promotion of flowering in the *METI* antisense plants. The flowering times of lines from the T2 and T3 generations of a single transgenic family, #10, were measured; Finnegan *et al*. (1996) reported the methylation level of T2 plants from family #10 to be approximately 18% of wildtype. Conditions for this experiment are shown in Table 3.1 (experiment 1).

In these conditions, C24 showed a strong vernalization response: the average flowering time for unvernalized plants was 63.5 days, while vernalized plants flowered in 21 days (Table 3.2, Figure 3.2). Plants from both the T2 and T3 generations of family #10 flowered significantly earlier than unvernalized C24 plants, although not as early as vernalized C24 plants (Table 3.2, Figure 3.2). Unvernalized plants from transgenic line T3#10.4, homozygous for the *METI* antisense construct, flowered, on average, 31 days earlier than wildtype, while unvernalized plants from line T3#10.1, which was

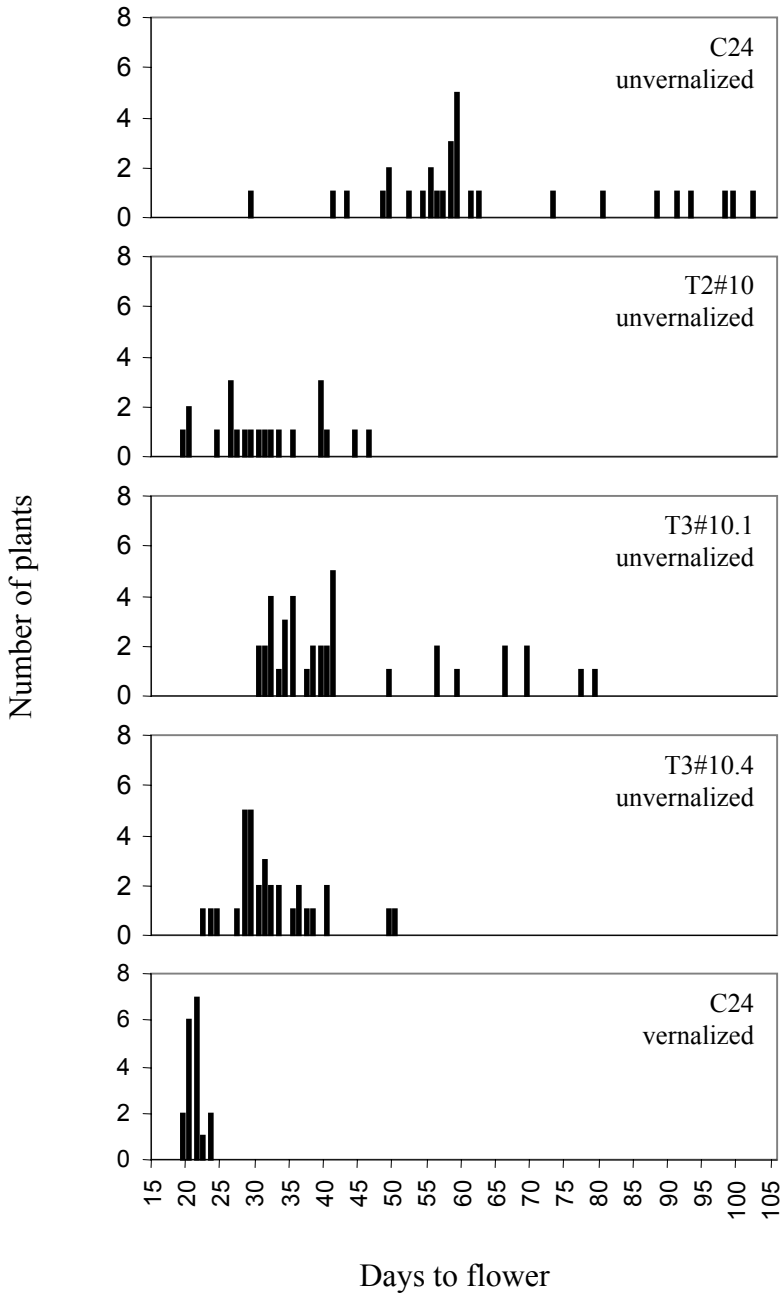
Plant Line	Unvernalized DTF ± s.e. (n)[a]	Vernalized DTF ± s.e. (n)
C24	63.46 ± 3.38 (29)	20.73 ± 0.27 (18)
T2#10	31.09 ± 1.71 (21)[†]	23.03 ± 1.1 (33)
T3#10.1	43.34 ± 2.29 (35)[†]	21.2 ± 1.06 (15)
T3#10.4	32.03 ± 1.12 (32)[†]	19.2 ± 1.06 (5)

Table 3.2: Days from germination to elongation of primary inflorescence for *METI* antisense plants grown in Experiment One. Growth conditions were short-day (eight hour) photoperiods under Philips TLMF 140W/33RD fluorescent lights.

[a]average days to flower ± standard error (number of plants)

[†]significantly different to C24 unvernalized at 1% (note that there is no significant difference in the flowering time of vernalized plants)

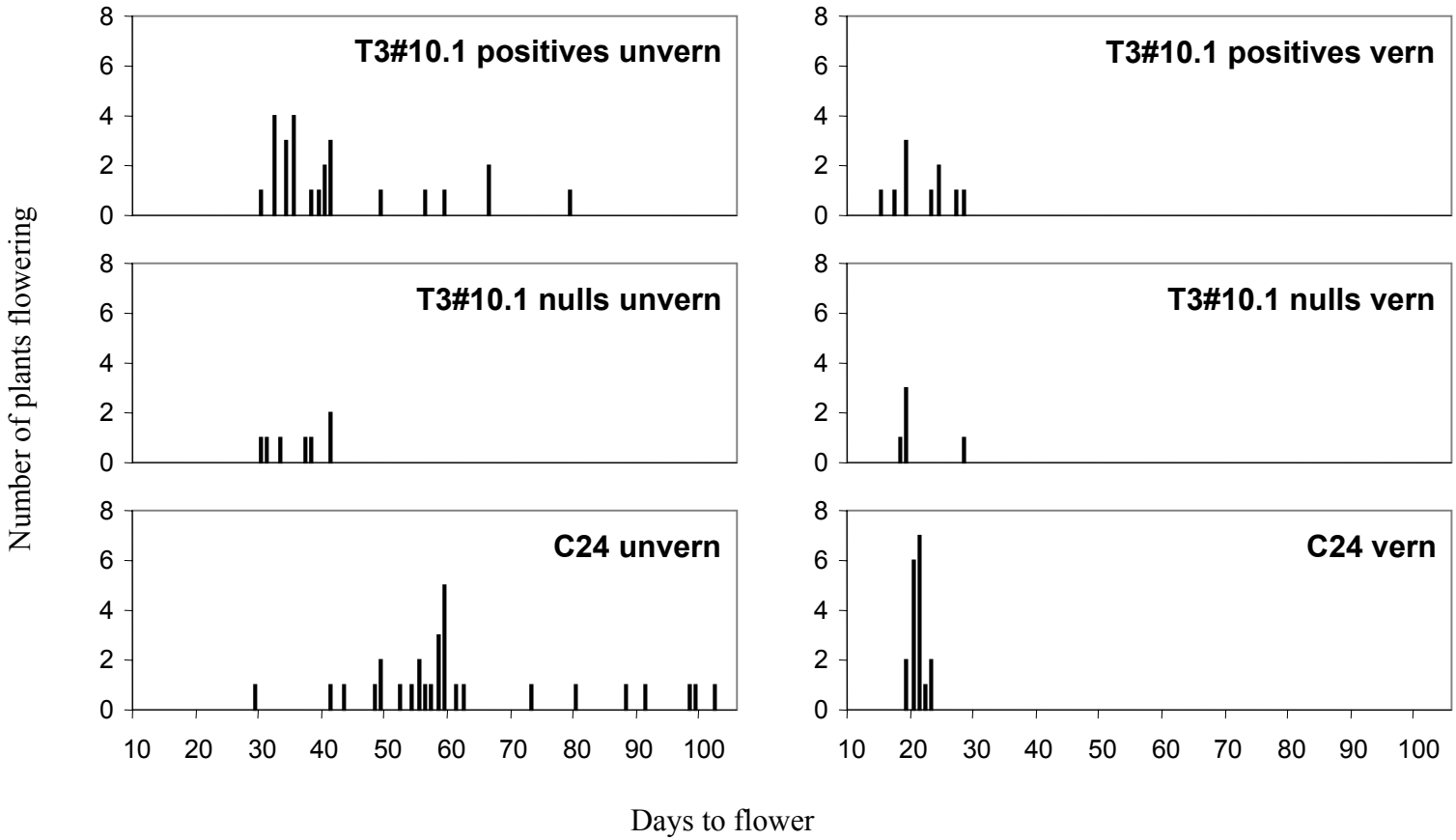
Figure 3.2: Promotion of flowering in plants from T2 and T3 generations of *METI* antisense transgenic family #10, relative to C24 wildtype control. T3 plants of line 10.1 are segregating for the presence of the transgene, while all T3 plants of line 10.4 contain the transgene. Flowering time of vernalized transgenic plants is not shown; these data appear in Table 3.2.


C24
unvernalized
T2#10
unvernalized
T3#10.1
unvernalized
T3#10.4
unvernalized
C24
vernalized
Number of plants
Days to flower
0
2
4
6
8
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105

segregating for the presence of the antisense transgene, flowered an average of 20 days early. Unvernalized plants from the T2 generation flowered 32 days earlier than wildtype; although this generation was segregating for the presence of the antisense transgene, by chance all plants in this experiment contained the *METI* antisense transgene.

The early flowering phenotype in these lines could be caused by demethylation resulting from the expression of the *METI* antisense transgene, or it could be due to a mutation resulting from insertion of the construct into a gene that delays flowering. As plants in line T3#10.1 were segregating for the antisense transgene, it was possible to compare the flowering times of sibling plants that did or did not contain the antisense transgene. Plants from line T3#10.1 were scored for the presence of the antisense construct. The presence of the transgene had no effect on flowering time, as plants that lacked the antisense construct flowered as early as those containing the construct (Figure 3.3). Previous measurements of antisense nulls from this line showed that cytosine methylation levels were reduced compared to wildtype, though higher than in plants containing the antisense construct (Finnegan *et al*., 1996). This indicates that cytosine demethylation, rather than the insertion of the transgene, is the cause of early flowering. These results also imply that sequences critical for early flowering are not readily remethylated: despite increased cytosine methylation in the antisense nulls, they still flower as early as their siblings that retain the antisense construct.

Figure 3.3: Flowering time of vernalized and unvernalized T3 plants of *METI* antisense line 10.1, comparing plants homozygous or hemizygous for the transgene (positives) with plants that do not contain the transgene (nulls). Lack of the transgene does not delay flowering.


T3#10.1 positives unvern
T3#10.1 positives vern
T3#10.1 nulls unvern
T3#10.1 nulls vern
C24 unvern
C24 vern
Number of plants flowering
Days to flower
0
2
4
6
8
10
20
30
40
50
60
70
80
90
100

3.3.2 Plants from four independent *METI* antisense families flower early

To confirm that demethylation is sufficient to cause early flowering, time to flowering was observed for lines from three other independent transgenic families transformed with the *METI* antisense construct: families #4, #22.6, and #39. Previous measurements of cytosine methylation on T2 plants from these families showed that methylation levels were reduced to 82% of wildtype for T2 plants from family #4, 68.5% for T2 plants from family #22.6 and 10-20% for T2 plants from family #39 (Finnegan *et al*., 1996; E.J. Finnegan, personal communication). This variation in degree of demethylation allowed an assessment of the quantitative effect of the extent of demethylation on flowering time. As for the previous experiment, plants were grown in 8 hour photoperiods, in order to maximize the difference in flowering time between unvernalized and vernalized plants, increasing the opportunity of detecting promotion of flowering in the *METI* antisense plants. Conditions for these experiments are shown in Table 3.1 (experiments 2, 3, 4 and 5).

The flowering time of T2 and T3 lines from family #4 were observed in experiments 2 and 3. In both experiments, unvernalized plants from the *METI* antisense lines flowered earlier than unvernalized C24 plants, though not as early as vernalized C24 plants (Tables 3.3 and 3.4). The type of lighting differed between the two experiments; in experiment 2, Phillips TLMF 140W/33RD fluorescent lights were used, and in experiment 3 Sylvania GTE F58W/133 fluorescent lights were used. Although the spectrum of light emitted by these lights is similar (Figure 3.1), flowering time differed between the two experiments.

Plant Line	Unvernalized DTF ± s.e. (n)[a]	Vernalized DTF ± s.e. (n)
C24	65.95 ± 1.12 (40)	27.2 ± 0.62 (32)
T2#4	56.3 ± 1.30 (39)[†]	27.2 ± 0.47 (38)
T3#4.2	58.6 ± 1.33 (38)[†]	30.8 ± 0.62 (33)[‡]
T3#22.6.11	54.0 ± 1.53 (36)[†]	32.5 ± 0.70 (37)[‡]

Table 3.3: Days from germination to elongation of primary inflorescence for *METI* antisense plants grown in Experiment Two. Growth conditions were short-day (eight hour) photoperiods under Philips TLMF 140W/33RD fluorescent lights.

[a]average days to flower ± standard error (number of plants)

[†]significantly different to C24 unvernalized at 1%

[‡]significantly different to C24 vernalized at 1%

Plant Line	Unvernalized DTF ± s.e. (n)[a]	Vernalized DTF ± s.e. (n)
C24	45.1 ± 1.52 (30)	23.4 ± 0.28 (36)
T3#4.2	43.1 ± 0.92 (37)	23.8 ± 0.29 (38)
T3#22.6.11	41.3 ± 0.80 (39)[§]	25.2 ± 0.38 (39)[‡]
T3#10.5	34.1 ± 0.70 (40)[†]	25.3 ± 0.37 (38)[‡]

Table 3.4: Days from germination to elongation of primary inflorescence for *METI* antisense plants grown in Experiment Three. Growth conditions were short-day (eight hour) photoperiods under Sylvania GTE F58W/133 fluorescent lights.

[a]average days to flower ± standard error (number of plants)

[†]significantly different to C24 unvernalized at 1%

[§]significantly different to C24 unvernalized at 5%

[‡]significantly different to C24 vernalized at 1%

Average flowering time for C24 under Sylvania lights (experiment 3, Table 3.4) was 45 days, while under Phillips lights (experiment 2, Table 3.3), average flowering time for C24 was 66 days. Promotion of flowering by both vernalization and *METI* antisense expression was greater in experiment 2 (under Phillips lights) than in experiment 3 (under Sylvania lights) (Tables 3.3 and 3.4). In experiment 2, vernalized C24 plants flowered in 41% of the time taken by unvernalized C24, while in experiment 3, vernalized C24 plants flowered in 51% of the time taken by unvernalized C24. Correspondingly, unvernalized plants from *METI* antisense line T3#4.2 flowered in 85% of the time taken by C24 plants in experiment 2, while in experiment 3, T3#4.2 plants flowered in 95.5% of the time taken by C24 plants.

T3 line #22.6.11 was grown in experiments 2, 3 and 5 (and in long days in experiment 7, which will be discussed later). Unvernalized plants from this line consistently flowered earlier than unvernalized C24 plants, but later than vernalized C24 plants (Tables 3.3, 3.4 and 3.6). Although the promotion of flowering due to demethylation in this line varied between experiments, it was roughly correlated with the extent of the C24 vernalization response. Vernalized C24 plants flowered in 39%, 41%, and 51% of the time taken by unvernalized C24 plants in experiments 5, 2 and 3 respectively. Corresponding figures for the flowering time of unvernalized T3#22.6.11 relative to unvernalized C24 were 86%, 81% and 91% for experiments 5, 2 and 3 respectively.

Line T3#39.35 was used in experiments 4 and 5. Unvernalized plants from this line showed the greatest promotion of flowering by demethylation, and flowered earlier than

both unvernalized and vernalized C24 plants (Tables 3.5 and 3.6). However, this did not demonstrate complete substitution of the vernalization response by demethylation, as, like all other *METI* antisense lines, line T3#39.35 flowered earlier when vernalized.

The four independent *METI* antisense trangenic lines have in common a significant reduction in cytosine methylation levels and an early flowering phenotype, reinforcing the conclusion that demethylation is sufficient to cause early flowering.

3.3.3 The promotion of flowering is correlated with the degree of demethylation

Lines from three of the four independent *METI* antisense transgenic families were grown in the same experiment (Experiment 5, Table 3.1), and this allowed a direct comparison of the extent of each family's early flowering response to demethylation. For families #10 and #22.6, lines which segregated for the antisense transgene, and which therefore differed in extent of demethylation, were included. In family #10 three copies of the antisense transgene were inserted at a single locus (Section 3.2.1). Three lines derived from T2 plant #10.1, which was hemizygous at the antisense transgene locus, were used: the T3 progeny (T3#10.1), in which the antisense transgene was segregating; line T4#10.1.4, of which all plants were homozygous for the antisense transgene; and antisense-null line T4#10.1.8. Family 22.6 possessed three co-segregating copies and one independently segregating copy of the antisense transgene. Two T3 lines from this family were used: line T3#22.6.11, which was homozygous for all copies of the antisense transgene, and line T3#22.6.9, which was homozygous for the three linked copies of the

Plant Line	Unvernalized DTF ± s.e. (n)[a]	Vernalized DTF ± s.e. (n)
C24	76.3 ± 2.28 (32)	34.4 ± 0.47 (37)
T3#10.5	43.1 ± 1.07 (38)[†]	30.4 ± 0.68 (37)[‡]
T3#39.35	30.6 ± 0.97 (36)[†]	24.6 ± 0.55 (37)[‡]
T3#10.1	56.4 ± 1.57 (39)[†]	28.1 ± 0.68 (38)[‡]
T3#10.1.8	41.1 ± 0.99 (38)[†]	25.9 ± 0.61 (37)[‡]

Table 3.5: Days from germination to elongation of primary inflorescence for *METI* antisense plants grown in Experiment Four. Growth conditions were short-day (eight hour) photoperiods under Osram 58W/21-84O fluorescent lights.

[a]average days to flower ± standard error (number of plants)

[†]significantly different to C24 unvernalized at 1%

[‡]significantly different to C24 vernalized at 1%

Plant Line	Unvernalized DTF ± s.e. (n)[a]	Vernalized DTF ± s.e. (n)	Methylation[b]
C24	86.6 ± 1.53 (36)	33.8 ± 0.91 (34)	
T3#10.1	58.2 ± 2.50 (20)[†]	28.0 ± 1.41 (18)[‡]	19.4 ± 4.2
T4#10.1.4	47.3 ± 2.4 (18)[†]	26.3 ± 1.13 (17)[‡]	20.1 ± 1.8
T4#10.1.8	54.6 ± 1.94 (20)[†]	27.8 ± 0.98 (20)[‡]	34.6[c]
T3#22.6.9	79.0 ± 2.31 (38)[§]	37.7 ± 0.91 (40)[‡]	56.9 ± 0.8
T3#22.6.11	75.0 ± 2.51 (40)[†]	34.8 ± 0.97 (37)	45.6 ± 3.2
T3#39.35	30.5 ± 1.02 (18)[†]	19.7 ± 0.73 (17)[‡]	32.2 ± 1.7

Table 3.6: Days from germination to elongation of primary inflorescence, and level of cytosine methylation, for *METI* antisense plants grown in Experiment Five. Growth conditions were short-day (eight hour) photoperiods under Osram 58W/21-84O fluorescent lights.

[a]average days to flower ± standard error (number of plants)

[b]percentage of cytosine methylation relative to C24 wildtype ± standard error

[c]average of two measurements

[†]significantly different to C24 unvernalized at 1%

[§]significantly different to C24 unvernalized at 5%

[‡]significantly different to C24 vernalized at 1%

transgene, and segregating at the locus of the unlinked copy of the transgene. The T3 line #39.35 was also used; the number of copies and integration sites of the antisense transgene in this family is not known. After flowering time was recorded, plant material for each line was pooled, and the level of cytosine methylation was measured for DNA extracted from this material.

The antisense null line, T4 #10.1.8, flowered early relative to the C24 control, but later than its sibling line, T4#10.1.4, which was homozygous for the antisense transgene (Table 3.6). The methylation level of T4#10.1.8 (34.6%) was almost double that of T4#10.1.4 (20.1 ± 1.8 %), though still much less than C24 wildtype (Table 3.6). A similar, though less marked, correlation between demethylation and promotion of flowering was seen for lines from family #22.6: plants from line T3#22.6.9 had higher methylation levels and flowered slightly later than plants from line T3#22.6.11 (Table 3.6). These results suggest that greater demethylation may cause earlier flowering, and this is supported by results from other experiments. In experiment 1 (Table 3.2), cytosine methylation was measured for a subset of plants from both T3#10.1 and T3#10.4. Plants were chosen to represent the range of flowering times observed, and all plants chosen from the segregating T3#10.1 line contained the antisense construct. In line T3#10.4, which had the earliest average flowering time, the average methylation level was 17.14% of wildtype levels, while the average level for T3#10.1 was 42.23%. Although methylation levels were not measured for plants from other flowering time experiments, relative flowering times of plants from different families correlate with the reported relative methylation levels of those families (Finnegan *et al*., 1996, 1998b), so that plants

from families #10 and #39 flower earlier than plants from families #22.6 and #4.

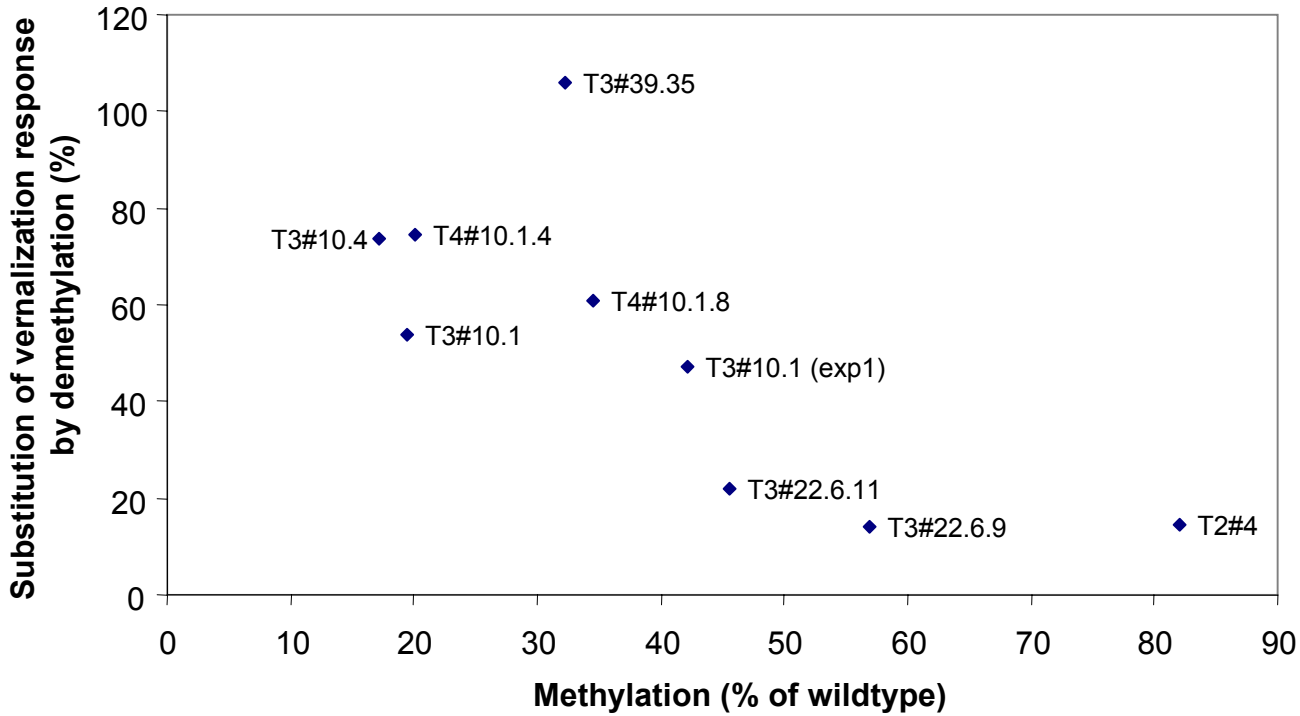
If the measure of promotion of flowering is expressed relative to the vernalization response of C24 for each experiment, it is possible to compare lines grown in different experiments. This was done for all lines for which cytosine methylation was measured: T3#10.1 and T3#10.4 (experiment 1); T2#4 (experiment 2); and all lines from experiment 7. A correlation between methylation level and promotion of flowering was found (Figure 3.4); methylation level accounts for over 50% of the variation in flowering time between methyltransferase antisense lines (r^2=0.544).

It should be noted that the methylation level for T3#10.1 plants from experiment 1 is more than double the level for T3#10.1 plants from experiment 5; the reason for this is unknown. All T3#10.1 plants from experiment 1 which were used to measure methylation contained the antisense construct, so the difference can not be ascribed to there being, by chance, a greater number of antisense nulls in the experiment 1 pool of plant material. However, the experiment 1 T3#10.1 plants flowered later, relative to C24, than the experiment 5 T3#10.1 plants, in line with the difference in methylation.

While the correlation between methylation level and promotion of flowering is strong, a considerable amount of variation in flowering time cannot be explained by the gross level of methylation. This is unsurprising, as changes in the gross level of methylation are an unsatisfactory molecular explanation for the features of vernalization, as will be discussed below. Specifically, the flowering times of lines T3#39.35 and T3#10.1, from experiment

Figure 3.4: Correlation of methylation level with promotion of flowering by demethylation in lines from families 4, 10, 22.6 and 39. Promotion of flowering is expressed as the proportion of the C24 vernalization response which is substituted for by the early flowering response to demethylation, calculated as follows:

$$\frac{\{\text{average FT (C24 unvernalized)} - \text{average FT (}\textit{METI}\text{ antisense line)}\} \times 100}{\{\text{average FT (C24 unvernalized)} - \text{average FT (C24 vernalized)}\}}$$


Substitution of vernalization response by demethylation (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Methylation (% of wildtype)
T3#39.35
T3#10.4
T4#10.1.4
T4#10.1.8
T3#10.1
T3#10.1 (exp1)
T3#22.6.11
T3#22.6.9
T2#4

5, are not predicted by the relationship between methylation and promotion of flowering shown in Figure 3.4. The methylation level of line T3#10.1 is substantially equivalent to that of line T4#10.1.4 in the same experiment; however, plants from line T3#10.1 flowered, on average, about eleven days later than plants from line T4#10.1.4. This may be due to differing distribution of demethylated sites in these two lines; hemimethylated sites in the T3#10.1 parent may have been remethylated or become fully demethylated in the T4#10.1.4 progeny, leaving the gross methylation levels approximately equivalent. If, by chance, sites important in the promotion of flowering have been fully demethylated in the DNA of T4#10.1.4 plants, flowering could be promoted more than in T3#10.1 plants, despite the similar methylation levels of the two lines. In the case of T3#39.35, flowering was much earlier than expected based on methylation. Again, it is possible that, by chance, sites important in promotion of flowering were demethylated in the DNA of this line, causing flowering to be earlier than in other lines with similar levels of methylation.

Although a broad correlation is seen between the extent of demethylation and promotion of flowering, this correlation does not hold for plants within a single transgenic line. Measurements of cytosine methylation on individual plants in lines T3#10.1 and T3#10.4 showed that within each line cytosine methylation ranged from 35.6 to 52.5% of wildtype and 11.2 to 22.9% of wildtype respectively. However, there was no relationship between methylation level and flowering time (Figure 3.5). The range of methylation levels within these lines was smaller than the range of levels between different families, making it less likely that a relationship between methylation and flowering would be detected.

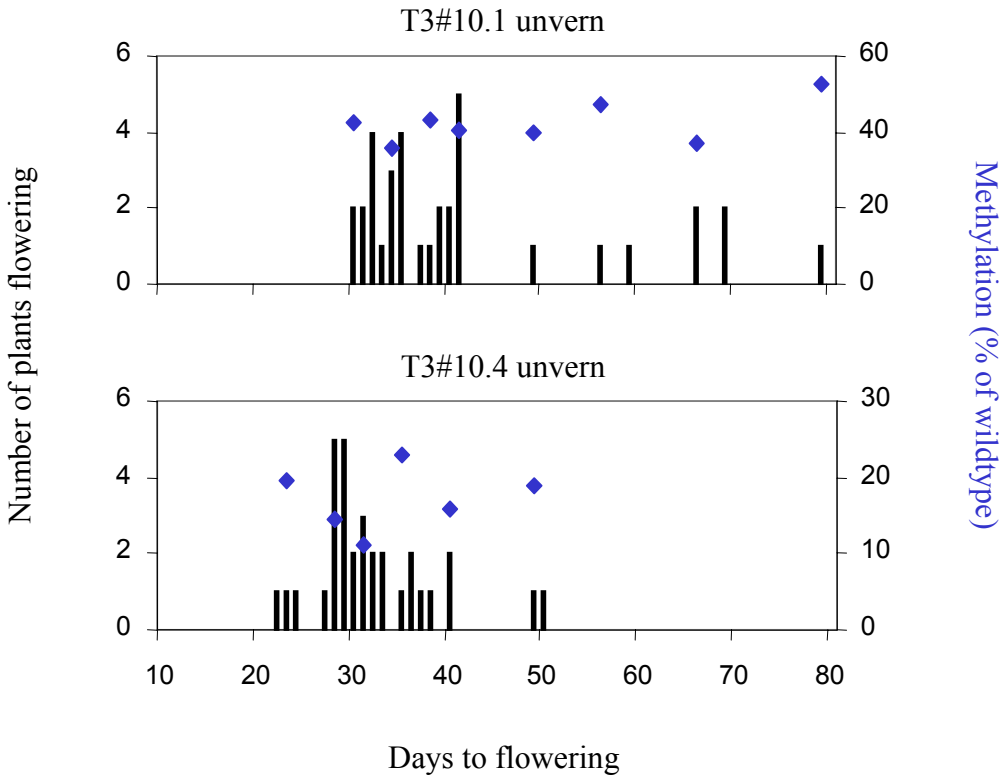


Figure 3.5: Flowering time of unvernalized T3 plants from lines 10.1 and 10.4, showing methylation levels of a subset of individual T3 plants. Flowering time of plants within these lines is not correlated with methylation level.

Also, as the demethylation caused by the *METI* antisense is stochastic and heritable, there should be more similarity in the spectrum of sequences demethylated in the DNA of closely related plants than of plants from different families. The persistence of the early flowering phenotype in family #10 plants that had lost the antisense transgene and showed increased methylation relative to their antisense-carrying siblings suggests that remethylation of sequences important in the early flowering phenotype is slow. Thus, if such sequences are demethylated in a particular plant, most of the progeny of that plant should inherit demethylation at that sequence and show a similar early flowering phenotype.

3.3.4 Effect of different light conditions and photoperiods on flowering time

Initial experiments to measure the effect of demethylation on flowering time were done in 8 hour days. In order to assess the effect of photoperiod on the flowering of plants with demethylated DNA, the flowering time of *METI* antisense transgenics was also measured in 16 hour days. Two experiments were done in 16 hour days; in one (Experiment 6, Table 3.1) light intensity was low, while in the other (Experiment 7, Table 3.1) light intensity was higher than usual.

C24 flowered earlier in 16 hour days than in 8 hour days, but still showed a large vernalization response. In the long day low light intensity conditions (experiment 6; Table 3.7), average flowering time for unvernalized C24 was approximately 30 days, and approximately 14 days for vernalized C24. In the long day high light conditions

Plant Line	Unvernalized DTF ± s.e. (n)[a]	Vernalized DTF ± s.e. (n)
C24	30.4 ± 1.27 (33)	14.4 ± 0.23 (40)
T3#4.2	38.4 ± 2.77 (17)[†]	12.8 ± 0.25 (39)[‡]
T3#22.6.11	28.4 ± 2.08 (22)[†]	16.4 ± 0.39 (38)[‡]
T3#10.5	21.42 ± 1.20 (12)[†]	14.5 ± 0.30 (40)

Table 3.7: Days from germination to elongation of primary inflorescence for *METI* antisense plants grown in Experiment Six. Growth conditions were long-day (sixteen hour) photoperiods under Philips TLMF 140W/33RD fluorescent lights, at low light intensity (80-100 μE).

[a]average days to flower ± standard error (number of plants)

[†]significantly different to C24 unvernalized at 1%

[‡]significantly different to C24 vernalized at 1%

Plant Line	Unvernalized DTF ± s.e. (n)[a]	Vernalized DTF ± s.e. (n)
C24	27.1 ± 0.69 (39)	10.0 ± 0.21 (38)
T3#4.2	26.5 ± 0.63 (38)	10.8 ± 0.29 (38)[§]
T3#22.6.11	21.8 ± 0.64 (35)[†]	11.8 ± 0.23 (39)[‡]
T3#10.5	21.7 ± 0.40 (39)[†]	13.1 ± 0.27 (40)[‡]

Table 3.8: Days from germination to elongation of primary inflorescence for *METI* antisense plants grown in Experiment Seven. Growth conditions were long-day (sixteen hour) photoperiods under Philips TLMF 140W/33RD fluorescent lights, at high light intensity (180-200 μE).

[a]average days to flower ± standard error (number of plants)

[†]significantly different to C24 unvernalized at 1%

[‡]significantly different to C24 vernalized at 1%

[§]significantly different to C24 vernalized at 5%

(experiment 7, Table 3.8), unvernalized C24 flowered in approximately 27 days, and in approximately 10 days when vernalized. Evidently photoperiod is more critical to flowering time than light intensity, at least in the range of light intensities and photoperiods used; although intensity in the high light conditions was double that in the low light conditions, only a small promotion of flowering was seen.

All plants from the *METI* antisense lines flowered earlier than C24 in long days, with the exception of plants from line T3#4.2 in low light conditions (Table 3.7). These plants flowered on average 8 days later than the C24 control in low light conditions, and only half a day earlier in high light conditions. It should be noted that in 8 hour days, the early flowering phenotype of this line was weaker than for other lines (Tables 3.3 and 3.4), and that the methylation level of this line is higher than for other *METI* antisense transgenic lines. This may explain the less uniform results for this line, as demethylation may be more variable at sequences critical for promotion of flowering.

Plants in different experiments using 8 hour days were grown under fluorescent lights which emitted three different spectra of wavelengths: Phillips TLMF 140W/33RD, Sylvania GTE F58W/133, and Osram 58W/21-84O (Figure 3.1). Of these experiments, light intensity was recorded for Experiments 3 (Sylvania), 4 and 5 (Osram) (Table 3.1). Light intensity was roughly comparable in all three experiments, though slightly higher in Experiment 3. Flowering was delayed under the Osram lights. In Experiment 3, average flowering time for C24 was 45 days (Table 3.4); this increased to 76 days in Experiment 4 (Table 3.5), and 87 days in Experiment 5 (Table 3.6). Vernalization response increased

under the Osram lights; vernalized C24 plants in Experiment 3 flowered 48% earlier than unvernalized plants; this figure increased to 55% and 61% in Experiments 4 and 5 respectively (Table 3.9). Two *METI* antisense transgenic lines were grown in both Sylvania and Osram lights: T3 plants of line #10.5 were grown in Experiments 3 and 4, and T3 plants of line #22.6.11 were grown in Experiments 3 and 5. Although flowering was later under Osram lights for both these lines, promotion of flowering in the *METI* antisense transgenics was greater, as a proportion of the C24 vernalization response, in the Osram lights than in the Sylvania lights (Table 3.9).

The vernalization response of C24, and the extent to which demethylation substituted for the vernalization response of C24 by promoting flowering, varied considerably between experiments. For T3 plants of line #22.6.11, the proportion of the C24 vernalization response for which promotion of flowering due to demethylation substituted was greater in experiments in which a larger C24 vernalization response was seen (Figure 3.6). However, for other lines which were grown in several experiments, no correlation was seen between C24 vernalization response and the early flowering response to demethylation (T3#10.5, T3#39.35; T3#4.2, T3#10.1; Figure 3.6).

3.3.5 Decreased methylation does not completely substitute for vernalization

In all light and photoperiod conditions, *METI* antisense transgenics show a vernalization response, demonstrating that demethylation does not fully substitute for vernalization or prevent the vernalization response (Tables 3.2 – 3.8). Within each experiment, time to

Lights	Sylvania	Osram	Osram
Experiment No.	3	4	5
C24 vernalization response	48.0%	54.8%	61.0%
T3#10.5	50.8%	79.3%	n.a.
T3#22.6.11	17.5%	n.a.	22.0%

Table 3.9: Promotion of flowering in *METI* antisense transgenic lines as a proportion of the C24 vernalization response under different light spectra. The C24 vernalization response was calculated as follows:

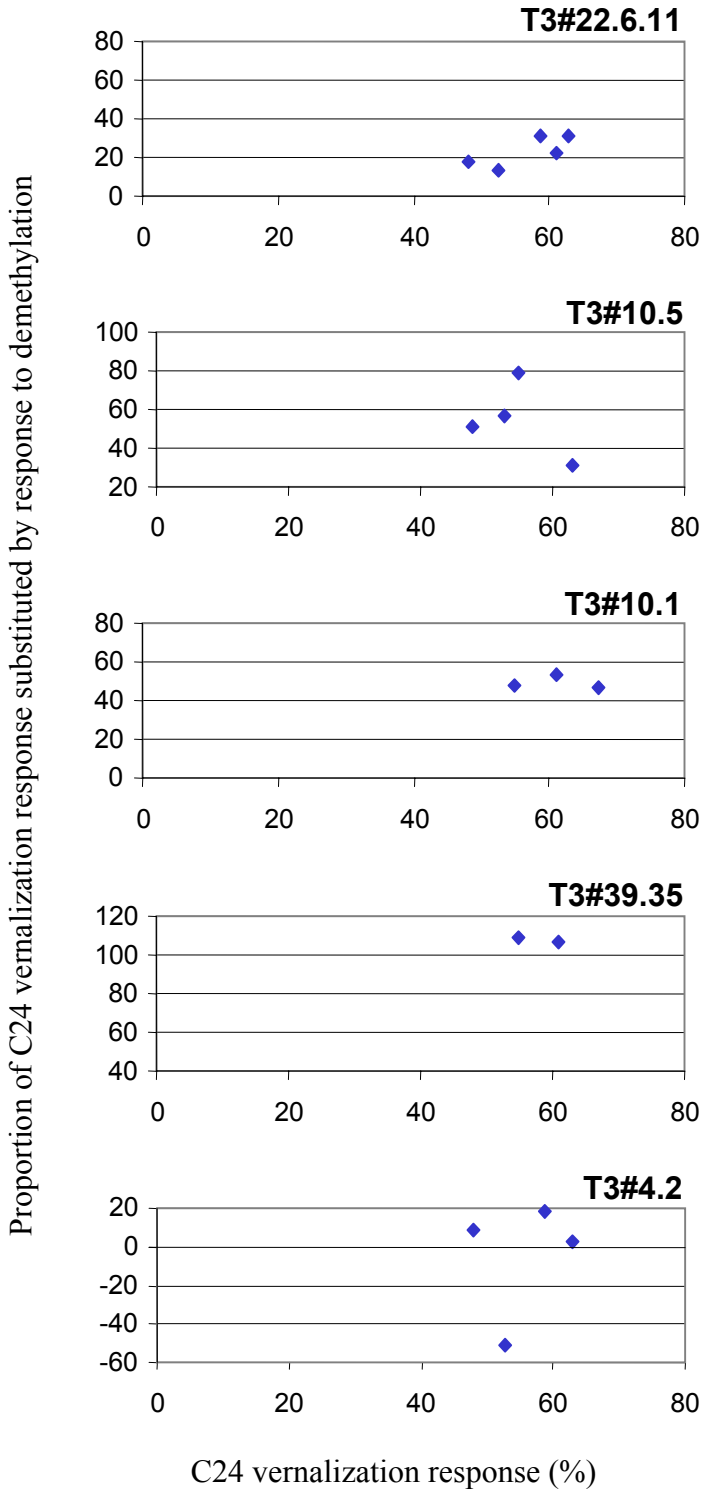
$$\frac{\{\text{average FT (C24 unvernalized)} - \text{average FT (}\textit{METI}\text{ antisense line)}\} \times 100}{\{\text{average FT (C24 unvernalized)}\}}$$

The proportion of the C24 vernalization response for which the early flowering response to demethylation substituted was calculated as follows:

$$\frac{\{\text{average FT (C24 unvernalized)} - \text{average FT (}\textit{METI}\text{ antisense line)}\} \times 100}{\{\text{average FT (C24 unvernalized)} - \text{average FT (C24 vernalized)}\}}$$

Figure 3.6: Relationship between the C24 vernalization response and the proportion of that response for which the early flowering response to demethylation substitutes. This proportion is calculated as follows:

$$\frac{\{\text{average FT (C24 unvernalized)} - \text{average FT (}\textit{METI}\text{ antisense line)}\} \times 100}{\{\text{average FT (C24 unvernalized)} - \text{average FT (C24 vernalized)}\}}$$


T3#22.6.11
80
60
40
20
0
0
20
40
60
80
T3#10.5
100
80
60
40
20
0
20
40
60
80
T3#10.1
80
60
40
20
0
0
20
40
60
80
T3#39.35
120
100
80
60
40
0
20
40
60
80
T3#4.2
20
0
-20
-40
-60
0
20
40
60
80
Proportion of C24 vernalization response substituted by response to demethylation
C24 vernalization response (%)

flowering was roughly similar for all vernalized plants, both C24 control plants and *METI* antisense transgenics, with the exception of T3 plants from line #39.35, discussed below. For all other lines, the combined effect of demethylation and vernalization resulted in flowering times similar to the flowering time of vernalized C24: lines which showed a large early flowering response to demethylation had a smaller response to vernalization, and vice versa. This suggests that demethylation may substitute for a part of the vernalization response.

The flowering behaviour of plants from line #39.35 differed from that of other lines. Unvernalized T3 plants from line #39.35 were alone among the *METI* antisense lines in flowering earlier than both unvernalized and vernalized C24 plants. The vernalization response of plants from line #39.35 was smaller than those seen for other lines in the same experiments (Tables 3.8 and 3.9); this observation matches the general trend that lines showing a large early flowering response to demethylation had a relatively small vernalization response. However, vernalized T3 plants of line #39.35 flowered earlier than vernalized C24 plants and vernalized plants from other antisense lines (Tables 3.7 and 3.8). This may indicate that the vernalization treatment was not saturating in C24, and that the combination of demethylation and vernalization was not saturating in the antisense lines. Alternatively, another factor that promotes flowering may be activated in line #39.35, perhaps due to specific demethylation sites in this line, or due to the insertion site of the *METI* antisense construct.

The flowering time of vernalized C24 and vernalized *METI* antisense plants differed by

small and sometimes statistically significant amounts, with the transgenics flowering either a few days earlier or later than wildtype. The interaction between demethylation and vernalization appeared to be related to light spectral quality. Under Osram 58W/21-84O fluorescent tubes, which delayed flowering, vernalized antisense plants flowered earlier than vernalized C24 plants (Tables 3.5 and 3.6), while, in general, under Phillips TLMF 140W/33RD or Sylvania F58W/133 tubes, vernalized antisense plants flowered later than vernalized C24 plants (Tables 3.2 – 3.4, 3.8 and 3.9).

Photoperiod had little or no effect on the interaction between demethylation and vernalization. Both 16 hour photoperiod experiments used Phillips TLMF 140W/33RD tubes. In these experiments, vernalized plants from the majority of *METI* antisense lines flowered later, on average, than vernalized C24 plants. This pattern was the same as that seen in 8 hour photoperiod experiments using Phillips TLMF 140W/33RD or Sylvania F58W/133 tubes.

3.3.6 Decreased DNA methylation (*ddm1*) mutant flowers early under short days

Plants from early generations of the *ddm1* mutant flowered slightly later than Columbia wildtype in 16 hour photoperiods (Kakutani *et al*., 1995). Flowering became progressively later in progeny from repeated selfing for six generations (Kakutani *et al*., 1996). The flowering time of plants from early generations of the *ddm1* mutant was observed in 16 hour and 8 hour photoperiods, to examine the difference in flowering time response to demethylation between *METI* antisense transgenics and the *ddm1* mutant.

In 16 hour days (Experiment 7, Table 3.1) Columbia plants flowered at an average of 14.5 days; this was reduced to 13 days by vernalization (Table 3.10). Both unvernalized and vernalized *ddm1* mutant plants flowered approximately 2 days later than Columbia wildtype (Table 3.10), confirming the reported observations. The vernalization responses of both Columbia and the *ddm1* mutant were small but statistically significant, with both flowering approximately one and a half days earlier, on average, when vernalized. When grown in 8 hour days under Sylvania F58W/133 fluorescent lights (Experiment 3, Table 3.1), Columbia plants flowered on average in 45 days, with the *ddm1* mutant flowering 3 days earlier (Table 3.10). In these conditions, the vernalization response of both Columbia and the *ddm1* mutant was increased compared to the response in 16 hour days. When flowering was delayed by the use of Osram 58W/21-84O fluorescent lights, both the vernalization response and the promotion of flowering in the *ddm1* mutant increased greatly (Experiments 4 and 5, Table 3.1). In Experiment 5, unvernalized Columbia plants flowered on average at 70 days, while vernalized plants flowered at approximately 41 days. Unvernalized *ddm1* mutant plants flowered on average at 49 days, and vernalization reduced this time to 37.5 days (Table 3.10).

The methylation level of the *ddm1* mutant, measured using plants grown in Experiment 5, was 19.1 ± 0.9 % of Columbia methylation. This is lower than the level of 28 % reported in Vongs *et al*. (1993).

Photoperiod (Experiment)	Plant Line	Unvernalized DTF ± s.e. (n)[a]	Vernalized DTF ± s.e. (n)
16 hours (7)	Columbia	14.5 ± 0.28 (36)	13.0 ± 0.17 (38)
	*ddm*1	16.4 ± 0.32 (39)[†]	15.0 ± 0.37 (40)[‡]
8 hours (3)	Columbia	41.0 ± 1.46 (39)	35.7 ± 1.77 (36)
	*ddm*1	37.2 ± 0.84 (39)	34.27 ± 0.9 (40)
8 hours (4)	Columbia	69.7 ± 2.11 (40)	48.8 ± 2.95 (37)
	*ddm*1	53.92 ± 2.06 (40)[†]	58.5 ± 2.09 (40)
8 hours (5)	Columbia	70.0 ± 3.35 (37)	40.7 ± 3.0 (35)
	*ddm*1	48.9 ± 2.25 (39)[†]	37.55 ± 2.79 (38)

Table 3.10: Days from germination to elongation of primary inflorescence for *ddm1* plants grown in long or short photoperiods, under Philips TLMF 140W/33RD (long photoperiods, experiment 7), Sylvania GTE F58W/133 (short photoperiods, experiment 3) or Osram 58W/21-84O (short photoperiods, experiments 4 and 5) fluorescent lights.

[a]average days to flower ± standard error (number of plants)

[†]significantly different to Columbia unvernalized at 1%

[‡]significantly different to Columbia vernalized at 1%

3.4 Discussion

The experiments reported here demonstrate that DNA demethylation is sufficient to cause early flowering in Arabidopsis. Early flowering does not depend on the position of integration of the transgene, as plants from independent transgenic families showed the early flowering response. Furthermore, the presence of the transgene is not required for the early flowering response; antisense nulls from the segregating T3 population of line #10.1 had reduced methylation levels compared to wildtype (Finnegan *et al*., 1996), and flowered as early as their antisense hemi- or homozygous siblings. T4 progeny of one antisense null segregant from the T3 generation of line #10.1, designated T3#10.1.8, had methylation levels of 34.6% of wildtype, and flowered in 63% of the time taken by wildtype plants. This confirms that the only requirement for early flowering in these plants was reduced methylation.

The demethylation-associated early flowering phenotype observed for the *METI* antisense plants is not necessarily connected with the vernalization response; however, a correlation was seen between early flowering due to demethylation and responsiveness to vernalization. Flowering time in Arabidopsis is influenced by environmental signals including photoperiod and light quality (Karlsson *et al*., 1993; Bagnall, 1993). Photoperiod and light intensity were varied, and the effect on flowering time was observed. In all growing conditions tested, a vernalization response was observed for C24 and for all *METI* antisense lines. Also in all these conditions, C24 plants with reduced methylation (*METI* antisense plants) flowered earlier than wildtype C24.

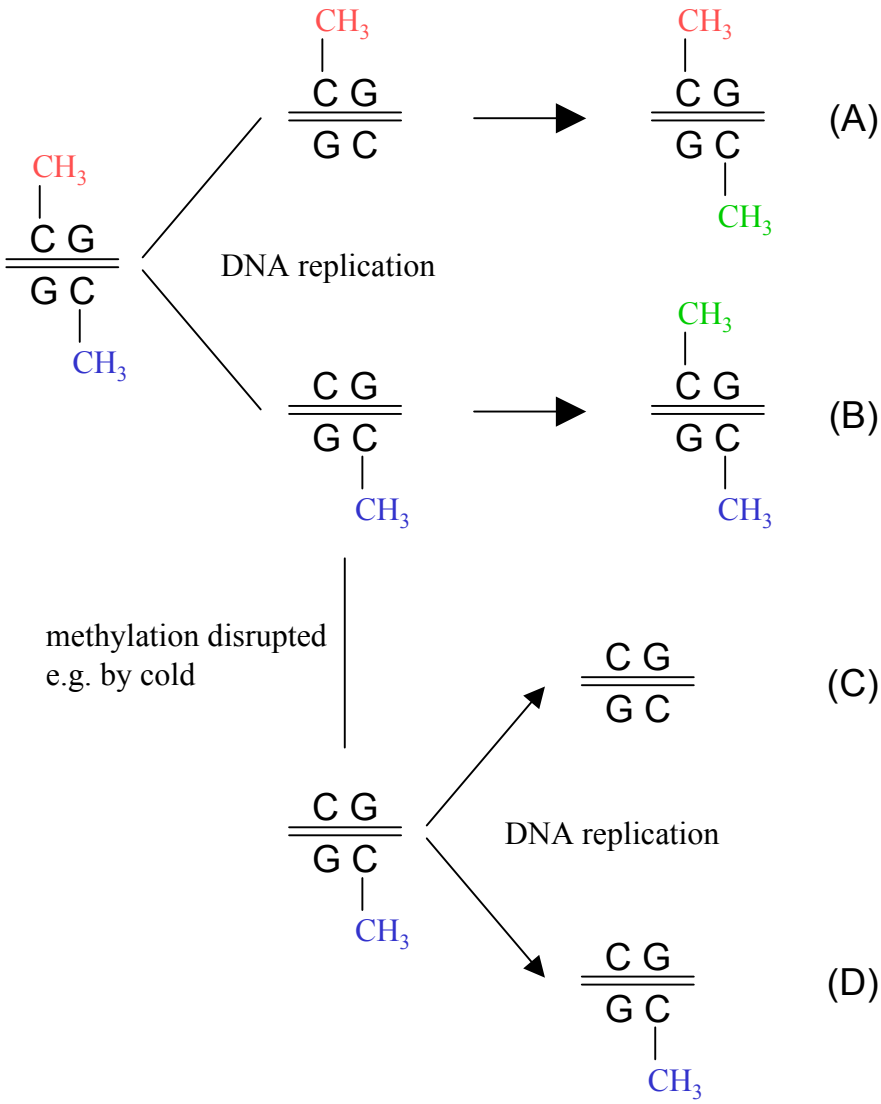
Columbia plants with reduced methylation – *ddm1* mutant plants – responded differently. In long day conditions, the vernalization response of both Columbia and *ddm1* was almost non-existent, and the *ddm1* mutant plants flowered slightly later than wildtype. However, in short day conditions, Columbia showed a greater vernalization response, and the *ddm1* mutant plants flowered significantly earlier than wildtype. These observations give a preliminary indication that the early flowering response to demethylation occurs only in conditions in which a vernalization response is seen, suggesting that demethylation may cause early flowering by the same mechanism as vernalization. Further experiments to examine the involvement of the vernalization response in demethylation-associated early flowering are reported in Chapter 4.

The level of methylation in different *METI* antisense lines ranged from approximately 18% (T3#10.4) to 82% (T2#4) of wildtype methylation (Table 3.7). Promotion of flowering was correlated with the extent of demethylation (Figure 3.4). If the promotion of flowering seen in these lines is due to the demethylation of specific regulatory DNA sequences, a greater extent of demethylation should increase the chance of these sequences being demethylated. This observation parallels the positive correlation between the length of the vernalizing treatment and the earliness of flowering in response to this low temperature treatment (Vince-Prue, 1975). As cell division is required for vernalization (Wellensiek, 1964), presumably a longer cold treatment would enable more cells to divide and enter the thermoinduced state. More cycles of cell division may be important for the effectiveness of cold-induced demethylation. If methyltransferases are cold sensitive, cold treatment may cause demethylation by uncoupling maintenance

methylation from DNA replication. After one cell cycle without maintenance methylation, previously methylated sites would be hemimethylated. Another cycle of cell division without methyltransferase activity would produce unmethylated sites on 2 of the 4 daughter molecules of DNA, and further cycles would dilute the original methylated residues (Figure 3.7), increasing the proportion of cells in adult plants containing demethylated sequences.

Vernalization leads to small, transient reductions in DNA methylation, yet has an effect on flowering time as great or greater than even the extensive demethylation reported here. Vernalization resulted in a transient decrease of methylation to 86.3 ± 0.9% of wildtype (Finnegan *et al*., 1998b). In contrast, the *METI* antisense line T3#39.35 had a methylation level of 32.2 ± 1.7% of wildtype, and unvernalized plants of this line flowered as early as vernalized wildtype. This may indicate that, while demethylation due to *METI* antisense occurs randomly, the demethylation occurring during the cold treatment is directed to sequences important in early flowering. Matsuo *et al*. (1998) suggest that, in *Xenopus* embryos, binding of transcription factors enhances demethylation of promoter sequences. A similar mechanism, in which binding of transcription factors to sequences important in early flowering enhances demethylation of those sequences, could account for the apparent specificity of vernalization-induced demethylation. The demethylation process reported by Matsuo *et al*. (1998) appears to be replication dependent, implying a passive mechanism. This fits with the hypothesis outlined in the preceding paragraph, that longer cold treatments lead to earlier flowering due to the greater number of cell cycles undergone without methylation of newly

Figure 3.7: A model for passive demethylation through disruption of maintenance methylation during DNA replication. If methylation is not disrupted, the pattern of DNA methylation is maintained (A and B). If methylation is disrupted, methylation is lost from one molecule (C); the other molecule, (D), retains one methylated cytosine. This site (on molecule D) may or may not be remethylated, depending on whether the disruption to methylation continues.


CH3
C G
G C
CH3
DNA replication
CH3
C G
G C
CH3
(A)
C G
G C
CH3
CH3
C G
G C
CH3
(B)
methylation disrupted
e.g. by cold
C G
G C
(C)
C G
G C
CH3
DNA replication
C G
G C
CH3
(D)

replicated DNA. Sequences critical for early flowering may be protected from remethylation after the cold treatment has ended, so that the return of normal methylation levels during growth at warmer temperatures does not impact on this response. Binding of transcription factors or other DNA binding proteins to such sequences could provide the mechanism for this protection.

Line T3#39.35 flowered earlier, relative to wildtype, than any other *METI* antisense line, despite having a higher methylation level than a number of lines from family #10. Also, although unvernalized plants from line T3#39.35 flowered as early as vernalized wildtype, vernalized T3#39.35 plants flowered even earlier. It is unlikely that the three week vernalization treatment used for these experiments saturated the vernalization response of C24, as L*er* plants containing a late flowering allele of *FRI*, and therefore having a similar *FRI* and *FLC* genotype to C24, required an 80 day cold treatment to saturate their vernalization response in short days (Lee and Amasino, 1995). Therefore the vernalization response of T3#39.35, and of other *METI* antisense lines, which all retained a vernalization response, may represent the additive effect of two non-saturating treatments leading to early flowering via the same pathway. An alternative possibility is that vernalization promotes flowering both through DNA demethylation and through another, so far uncharacterized effect. Thus, demethylation of DNA would substitute for only part of the effect of the cold treatment, leaving plants with a reduced response to cold treatment. Another alternative is that demethylation and vernalization lead to early flowering via two separate pathways, and that the early flowering seen in T3#39.35 and other *METI* antisense lines is due to the additive effect of these two pathways. The

specificity of the demethylation-related early flowering phenotype to the vernalization response is examined in Chapter 4.

The early flowering phenotype was transmitted to progeny, even when the *METI* transgene was not. While the absence of the transgene allowed methylation levels to increase slightly, methylation levels did not return to wildtype levels, suggesting that *de novo* methylation occurs slowly in Arabidopsis, and that methylation patterns are not reset between generations. Both line T4#10.1.8, which was antisense null and line T3#22.6.9, which was segregating for one of four copies of the antisense, had increased methylation levels relative to lines T4#10.1.4 and T3#22.6.11 respectively, both of which were homozygous for the antisense transgene at all integration sites. The flowering times of these lines (T4#10.1.8 vs. T4#10.1.4 and T3#22.6.9 vs. T3#22.6.11) were consistent with the correlation between demethylation and earliness of flowering shown in Figure 3.4. However, it should be noted that line T4#10.1.4, while remaining at the methylation level of T3#10.1, flowered much earlier than this line. This may be due to differing distribution of demethylation in the two lines; hemimethylation in the T3#10.1 parent may have been consolidated into full demethylation in the T4#10.1.4 progeny. Confirmation of these suggested explanations must await the isolation of sequences critical in the vernalization response, which will enable characterization of the methylation status of these sequences in vernalized and unvernalized plants, and in the demethylated *METI* antisense lines.

Differences in the flowering time response to demethylation of the ecotypes Columbia

and C24 reported in Section 3.3.6 are confirmed by the work of Ronemus *et al*. (1996), who transformed Columbia with a *METI* antisense construct similar to that used by Finnegan *et al*. (1996) in the C24 *METI* antisense lines used here. Ronemus *et al*. (1996) observed that transgenic lines showing DNA demethylation flowered later than wildtype Columbia in long day conditions. This difference, and the different response to vernalization of C24 and Columbia, may be explained by allelic differences at the *FRI* and *FLC* loci. These genes regulate flowering time in Arabidopsis; in combination, late flowering alleles at these loci cause extreme lateness (reviewed in Koornneef *et al*., 1998a). Plants carrying the late flowering allele of *FRI* show a large response to vernalization in both long and short photoperiods (Lee and Amasino, 1995). C24 plants carry the late flowering *FRI* allele, while Columbia plants carry the *FRI* allele for early flowering (Burn *et al*., 1993b; Lee *et al*., 1993), likely to be a null allele (Michaels and Amasino, 1999). C24 plants flower early in response to vernalization in both long and short photoperiods, and will likewise respond to demethylation in both long and short photoperiods. In long photoperiods, Columbia plants do not flower early in response to vernalization (Lee and Amasino, 1995; this chapter) or to demethylation. Columbia carries the late flowering allele of *FLC*, which, in the absence of a late flowering allele of *FRI*, delays flowering slightly in short days, but not long days (Sanda and Amasino, 1996; Michaels and Amasino, 1999), causing Columbia to be dependent on vernalization for early flowering only in short days, and likewise to show an early flowering response to demethylation only in short days. C24 carries an early flowering allele of *FLC*, which suppresses the late flowering phenotype caused by the late *FRI* allele it carries (Sanda and Amasino, 1995). As *FRI* and *FLC* are major genes regulating flowering time, it is

tempting to attribute differences in flowering behaviour of C24 and Columbia to differences at these loci. However, it is also possible that allelic differences between C24 and Columbia at other loci are responsible for their differences in response to vernalization and demethylation in long or short photoperiods.