Chapter Four

Effect of demethylation on the flowering time of late flowering mutants of *Arabidopsis thaliana*

4.1 Introduction

Flowering time in *Arabidopsis thaliana* is influenced by environmental stimuli, particularly photoperiod and vernalization. Different ecotypes of Arabidopsis vary in their flowering time and response to these environmental stimuli. In a survey of 32 ecotypes, 30 flowered early in response to long days, and 16 of the 30 also flowered early in response to vernalization (Karlsson *et al*., 1993). Two ecotypes did not flower after 110 days, regardless of photoperiod and vernalization, but the remaining 30 ecotypes flowered eventually even in short days (Karlsson *et al*., 1993). This is consistent with previous findings that most ecotypes of Arabidopsis have at most a quantitative requirement for long days and vernalization (Napp-Zinn, 1985).

A number of genes involved in the control of flowering time in Arabidopsis have been identified, both by analysis of natural variation in flowering time among ecotypes, and by characterization of late and early flowering mutants (reviewed in Koornneef *et al*., 1998a; Levy and Dean, 1998). Late flowering Arabidopsis mutants at loci *CO*, *GI* and *LD* were first isolated by Redei (1962) in the Columbia background. Mutants at the same and other loci were isolated in the L*er* background by Koornneef *et al*. (1991) and in the Wassilewskija (Ws) background by Lee *et al*. (1994b). The set of late flowering mutants

co, gi, ld, fd, fe, ft, fy, fca, fha, fpe, fve and *fwa* isolated in these studies was separated into classes based on responsiveness to photoperiod and vernalization, and on epistatic interactions (Koornneef *et al*., 1991, 1998b). On the basis of this classification, it has been proposed that there are several pathways promoting flowering, each of which is blocked by mutations in genes for a particular mutant class (Burn *et al*., 1993a and b; Koornneef *et al*., 1998a and b; Levy and Dean, 1998).

Mutants at loci *FCA*, *FVE*, *FPA*, *FY* and *LD* (Class 1, Figure 4.1) flower later than wildtype in long day conditions, and much later in short day conditions, exhibiting an increased photoperiod response. These mutants also show a larger response to vernalization than wildtype L*er* (Redei, 1962; Koornneef *et al*., 1991; Bagnall, 1993; Lee *et al*., 1994b). The increased photoperiod and vernalization response of the Class 1 late flowering mutants suggests that they are blocked in an autonomous pathway to flowering, making the transition to flowering more dependent on another pathway(s), regulated by photoperiod or vernalization (Figure 4.1; Dennis *et al*., 1996; Koornneef *et al*., 1998a).

Mutants at loci *FE*, *FT*, *FD*, and *FWA* (Class 2, Figure 4.1) flower later than wild type in both long and short days, and have a somewhat reduced photoperiod response, compared to wildtype. The vernalization response of mutants of this class is non-existent or small, similar to that of the wildtype L*er* (Koornneef *et al*., 1991, Bagnall, 1993). Class 2 late flowering mutants are thought to be blocked in a photoperiod promotion pathway (Figure 4.1; Koornneef *et al*., 1998a). Although these mutants show only a small vernalization response, this does not imply that they are blocked in a vernalization promotion pathway,

Figure 4.1: A model for the transition from vegetative growth to flowering, showing the role of the floral repressor *FLC* in the autonomous and vernalization pathways. Genes in the autonomous pathway (*FCA*, *LD*, *FVE*, *FPA*) act to promote flowering by down-regulating *FLC* expression, as does the *VRN2* gene in the vernalization pathway (Sheldon *et al*., 1999; Michaels and Amasino, 1999). The *FLC* gene product appears to suppress the effect of gibberellic acid in promoting flowering (Sheldon *et al*., 1999). There is no evidence for interactions between *FLC* and genes in the flowering pathway(s) regulated by photoperiod and light quality; these genes (*FE*, *FT*, *FD*, *FWA*, *CO*, *GI*, *FHA*) may interact with another, as yet unknown floral repressor.

VEGETATIVE

Light Quality and Photoperiod

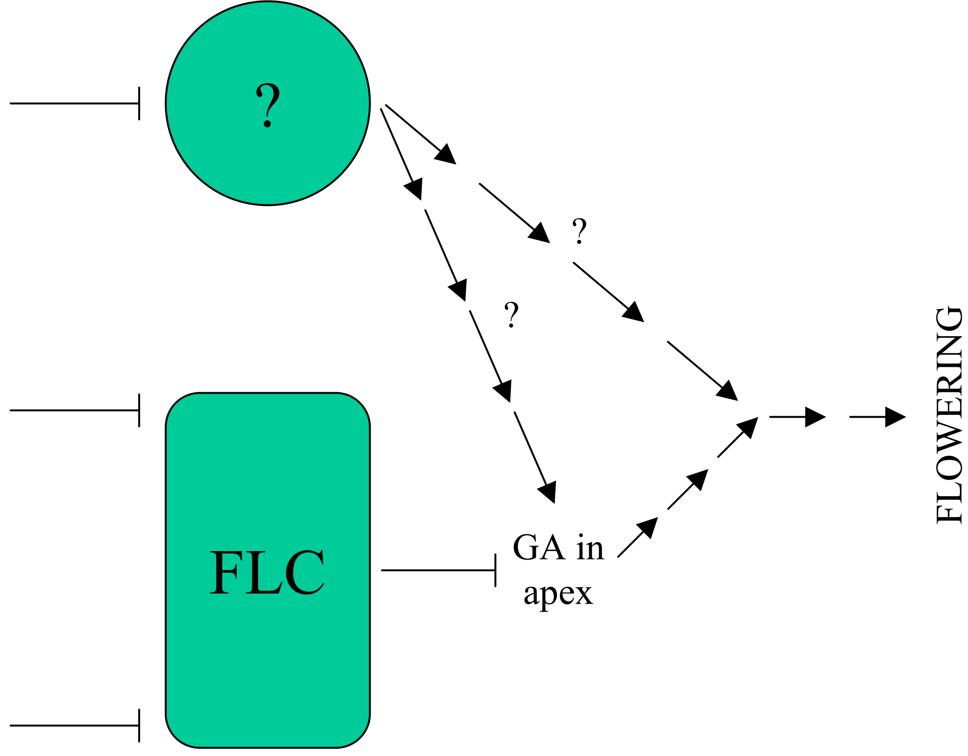
Class 2 and 3
FE, FT, FD,
FWA, CO,
GI, FHA

Autonomous

Class 1
FCA, LD,
FVE, FPA

Vernalization

VRN2



as long vernalization treatments result in early flowering (Chandler and Dean, 1994) and double mutants between these mutants and Class 1 (vernalization responsive) mutants are responsive to vernalization (Koornneef *et al*., 1998a).

In the third group of mutants, *co*, *gi*, and *fha* (Class 3, Figure 4.1), photoperiod and vernalization responsiveness is reduced relative to wildtype (Koornneef *et al*., 1991; Bagnall, 1993), suggesting an inability to respond to these stimuli. These mutants fall into the same epistatic group as the Class 2 mutants (Koornneef *et al*., 1998b), suggesting that they may also act in the photoperiod response pathway. However, *CO* may be involved in GA signal transduction (Putterill *et al.*, 1995). The *co* mutant is not rescued by GA application, and double mutants between *co* and *ga1-3* or *gai*, mutants affected in GA biosynthesis or signal transduction, show GA deficient phenotypes including later flowering (Putterill *et al*., 1995).

Another class of mutants affected in flowering time, *vrn1* and *vrn2*, were isolated in the *fca* mutant background, where they show a large reduction in vernalization response (Chandler *et al*., 1996). These mutants are likely to be blocked in a vernalization promotion pathway, although *vrn2*, which delays the flowering of *fca* whether vernalized or unvernalized, may also be blocked in another pathway (Chandler *et al*., 1996; Figure 4.1).

Results reported in Chapter Three showed that decreased DNA methylation led to early flowering in two vernalization responsive ecotypes of Arabidopsis, C24 and Columbia,

especially in short days (also reported in Finnegan *et al*., 1998b). Sheldon *et al*. (1999) isolated a floral repressor *FLF* (*FLC*), which is down-regulated in vernalized plants and in C24 plants with methylation decreased by *METI* antisense**.** As methylation levels are decreased by cold treatment (Burn *et al*., 1993a; Finnegan *et al*., 1998b), it appears likely that vernalization promotes flowering through DNA demethylation, which leads to down-regulation of *FLF*.

FLF mRNA levels are increased relative to wildtype in the Class 1 late flowering mutants *fca*, *ld*, *fve*, and *fpa*, and in the *fca vrn2* mutant, indicating that the products of these genes, which are predicted to operate in the autonomous (*FCA, LD, FVE, FPA*) and vernalization (*VRN2*) pathways to flowering (Figure 4.1), down-regulate *FLF* expression (Sheldon *et al*., 1999). Mutations in genes in the photoperiod promotion pathway (Class 2: *FE*, *FT*, *FD*, *FWA*) do not affect *FLF* expression (Sheldon *et al*., 1999), indicating that this pathway does not promote flowering by down-regulating *FLF*. If demethylation causes early flowering by decreasing *FLF* expression, no early flowering response to demethylation should be seen in late flowering mutants which do not respond to vernalization. Conversely, late flowering mutants in which *FLF* levels are increased and which respond to vernalization should flower early when DNA methylation levels are reduced.

This prediction was tested by crossing the *METI* antisense transgene into two late flowering mutants: *fca* , which flowers early in response to vernalization, and *fe*, which does not flower early when vernalized. F2 plants homozygous for the late flowering

mutation and carrying the *METI* antisense transgene were identified, and the flowering time of the progeny of these plants was measured. The two mutants responded differently to demethylation: demethylated *fca* lines flowered early relative to control plants, while demethylated *fe* lines flowered no earlier than control plants.

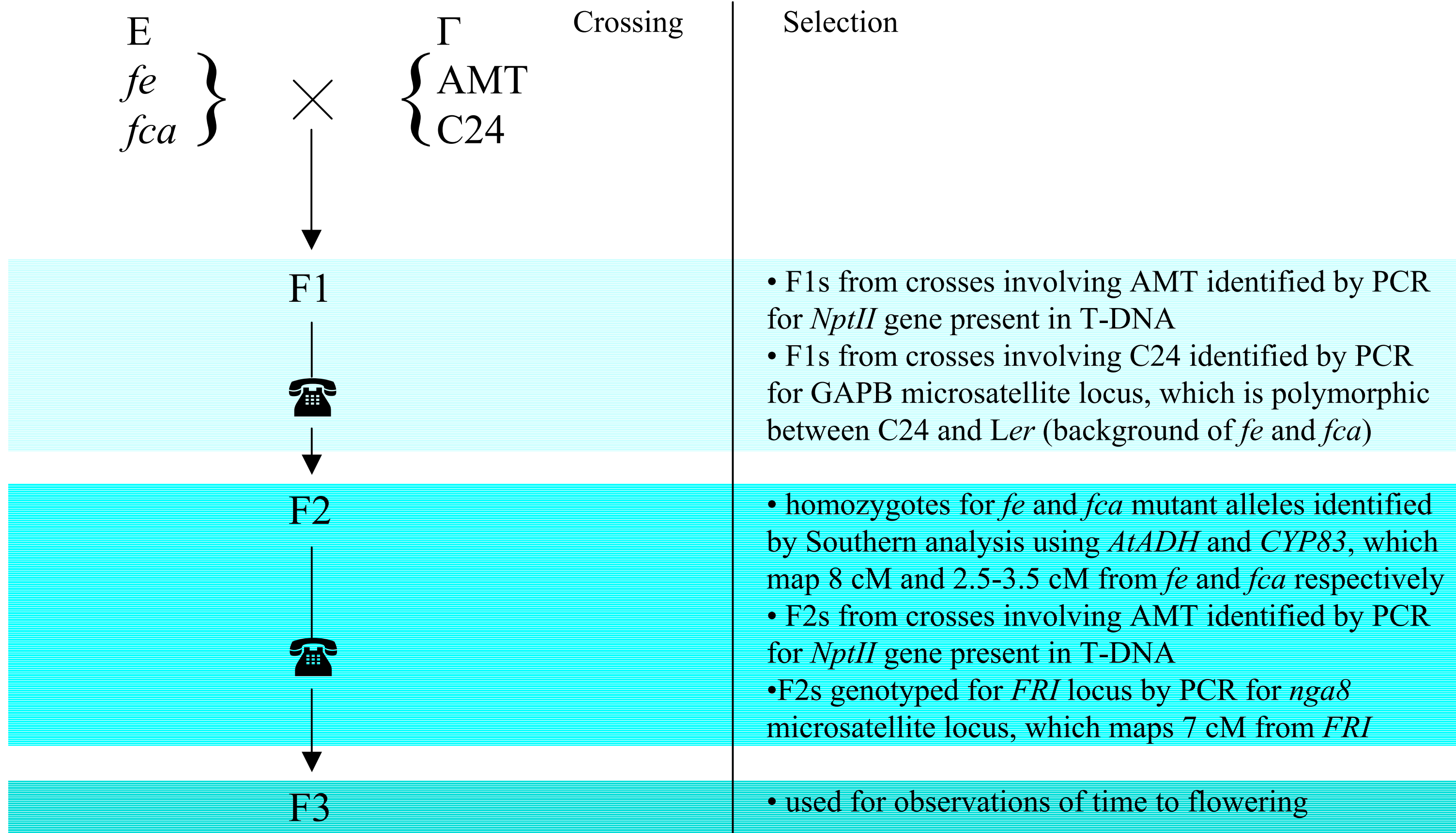
4.2 Materials and Methods

4.2.1 Introduction of a *METI* antisense construct into late flowering mutant backgrounds

Landsberg *erecta* (L*er*) and the late flowering mutants in this background are very difficult to regenerate from callus tissue, and therefore very difficult to transform by the *Agrobacterium*-mediated tissue culture method commonly used for Arabidopsis (E.J. Finnegan, personal communication). Multiple attempts were made to transform *fca*, *fe*, and L*er* by vacuum infiltration (section 2.7; Bechtold *et al*., 1993), but these were unsuccessful. Therefore the antisense construct was crossed into the *fca* and *fe* backgrounds. The process of crossing and selection of the appropriate lines for flowering experiments is shown in Figure 4.2.

Two sets of crosses were made for each mutant: the homozygous late flowering mutant with the homozygous *METI* antisense line T3#10.5 as pollen donor; and the homozygous late flowering mutant with C24, the wildtype control for the antisense plants. The F1 progeny from crosses with T3#10.5 were screened for the *NptII* gene linked to the *METI*

Figure 4.2: Crossing and selection procedures for generating lines containing either the *fe* or *fca* mutation, with or without the *METI* antisense construct from T3 line 10.5 (AMT). Each late flowering mutant was crossed to both C24 and to the AMT line.



E
fe
fca
Γ
AMT
C24
Crossing
Selection
F1
• F1s from crosses involving AMT identified by PCR for NptII gene present in T-DNA
• F1s from crosses involving C24 identified by PCR for GAPB microsatellite locus, which is polymorphic between C24 and Ler (background of fe and fca)
F2
• homozygotes for fe and fca mutant alleles identified by Southern analysis using AtADH and CYP83, which map 8 cM and 2.5-3.5 cM from fe and fca respectively
• F2s from crosses involving AMT identified by PCR for NptII gene present in T-DNA
•F2s genotyped for FRI locus by PCR for nga8 microsatellite locus, which maps 7 cM from FRI
F3
• used for observations of time to flowering

antisense construct, by PCR using DNA prepared from a single leaf from each plant, as described in Section 2.2.4; the conditions for this PCR analysis are described in Section 2.3.2. Seed was collected from plants containing the *Npt*II gene. The F1 progeny from crosses with C24 were screened by PCR with the CAPS (Cleaved Amplified Polymorphic Sequence) marker GAPB (Konieczny and Ausubel, 1993); conditions for this PCR analysis are described in section 2.3.4. The amplification product from this primer pair, when cleaved with *Dde*I, distinguishes between L*er* and C24 in a codominant manner, allowing F1s to be identified easily (Figure 4.3). Seed was harvested from plants heterozygous for L*er* and C24 at the GAPB locus.

F2 plants homozygous for the relevant mutant locus were selected by genomic Southern analysis, using DNA fragments of genes which map close to the mutant loci as RFLP markers. The CTAB-CsCl DNA isolation protocol, conditions for restriction digests and Southern blotting method are described in Sections 2.2.1 and 2.3.1. For the *fe* late flowering mutant lines, a 3.7 kb *Sac*I genomic DNA fragment containing the Arabidopsis *ADH* gene (Genbank accession M12196; Chang and Meyerowitz, 1986; Dolferus *et al*, 1990) was used. *AtADH* maps 8cM from the *FE* locus, according to the classical genetic map maintained by David Meinke (http://genome-www3.stanford.edu/cgi-bin/AtDB/Genintromap). An RFLP between L*er* and C24 DNA cleaved with *Eco*RV was found for *AtADH* (Figure 4.4a). For the *fca* late flowering mutant lines, a 1.6 kb *Sal*I-*Not*I cDNA fragment of the Arabidopsis CYP83 gene (Genbank accession U69134; Bilodeau *et al*., 1999), which maps 2.5-3.5 cM from the *FCA* locus (P. Bilodeau, personal communication), was used. An RFLP between L*er* and C24 DNA cleaved with *Xba*I was

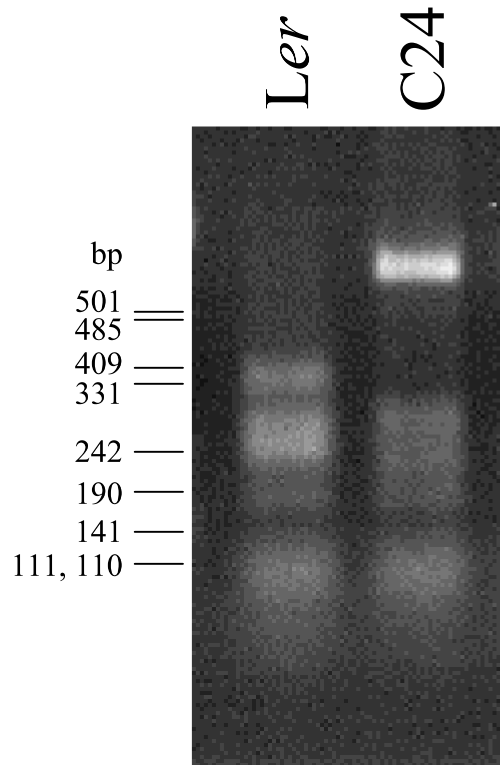
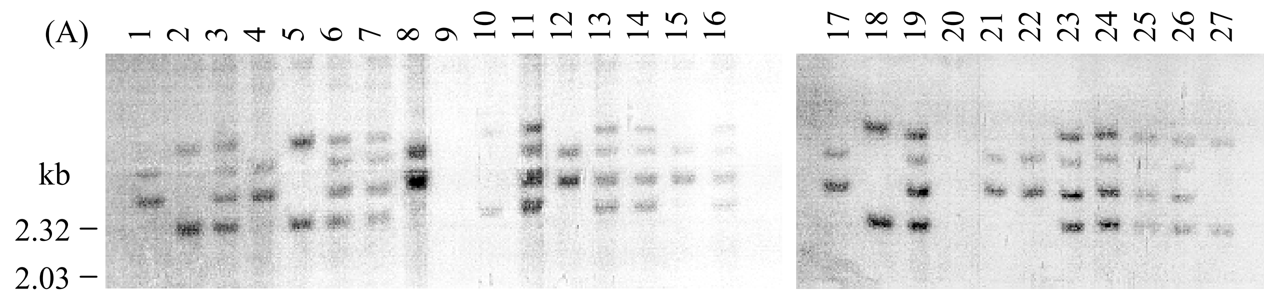


Figure 4.3: Polymorphism between C24 and L*er* using the GAPB marker. The product of PCR using primers for the GAPB microsatellite locus (Konieczny and Ausubel, 1993), after cleavage with *Dde*I, differs in size between C24 and L*er*.


(A)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
kb
2.32
2.03

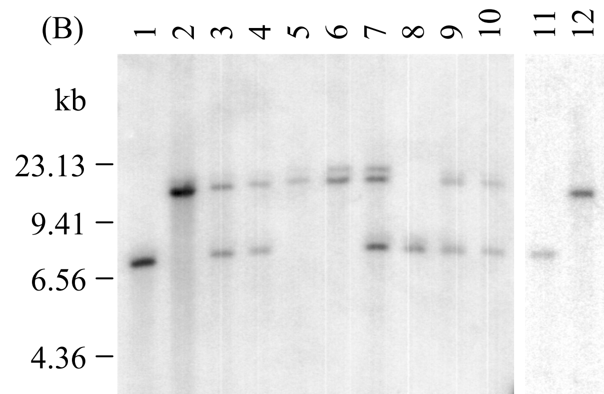

(B)
1
2
3
4
5
6
7
8
9
10
11
12
kb
23.13
9.41
6.56
4.36

Figure 4.4: Identification of F2 plants from crosses between *fe* or *fca*, and C24 or *METI* antisense line T3#10.5, homozygous for the mutant locus.

(A) Southern blot analysis of *Eco*RV cleaved plant DNA, hybridized with a 3.7 kb genomic DNA fragment containing the Arabidopsis *ADH* gene, linked to the *fe* locus. Lanes 1 and 17 contain L*er* DNA, while lanes 2 and 18 contain C24 DNA. Lanes 3 to 16 contain DNA from F2 plants 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 21 and 24, from crosses between *fe* and *METI* antisense T3 line 10.5 (AMT). F2 plants *fe*-AMT # 2, 7, 11 and 21 are homozygous for the L*er* AtADH allele. Lanes 19 to 27 contain DNA from F2 plants 1, 2, 3, 4, 5, 7, 8, 9 and 10, from crosses between *fe* and C24. F2 plants *fe*-C24 # 3 and 4 are homozygous for the L*er* AtADH allele.

(B) Southern blot analysis of *Xba*I restricted plant DNA, probed with a 1.6 kb cDNA fragment of the Arabidopsis CYP83 gene, linked to the *fca* locus. Lanes 1 and 2 contain C24 and L*er* DNA respectively. Lanes 3, 4, 11 and 12 contain DNA from F2 plants 2, 4, 17 and 21, from crosses between *fca* and *METI* antisense T3 line 10.5 (AMT); F2 plant *fca*-AMT #21 is homozygous for the L*er* CYP83 allele. Lanes 5 to 10 contain DNA from F2 plants 1, 4, 6, 10, 19 and 24, from crosses between *fca* and C24; F2 plants *fca*-C24 # 1 and 4 are homozygous for the L*er* CYP83 allele.

found for CYP83 (Figure 4.4b). PCR for the *Npt*II gene, as described in Chapter Two, was used to ensure that F2 progeny from crosses with T3#10.5 had inherited the *METI* antisense transgene.

The genotype of F2 plants at the *FRI* locus was determined by PCR for the microsatellie locus *nga8* (Bell and Ecker, 1994), which maps approximately 7 cM from the *FRI* locus (R. Amasino, personal communication). Reaction conditions for *nga8* PCR are described in Section 2.3.3. The *nga12* microsatellite marker (Bell and Ecker, 1994) maps approximately 3 cM from *FRI*, and therefore would have been more appropriate to use than *nga8*. However, in initial tests of the primers, it was found that *nga12* primers amplified a fragment from L*er* but not from C24, making it impossible to discriminate between plants homozygous for the L*er* allele of *nga12* and plants heterozygous at this locus.

F3 lines derived from crosses with the *METI* antisense line T3#10.5 were designated *fe*-AMT (antisense methyltransferase) and *fca*-AMT depending on the late flowering mutant parent. F3 lines derived from crosses with C24 were designated *fe*-C24 and *fca*-C24 depending on the late flowering mutant parent.

4.2.2 Experiments to measure flowering time

Conditions for the flowering time experiment were as described in Section 2.1. For most lines, 40 tubes were prepared for both control and vernalization treatments. In the case of

fca-AMT line #21, which is heterozygous at the *FRI* locus, 200 tubes were prepared for both unvernalized and vernalized plants, in order to ensure a sufficient number of homozygotes for *FRI*-L*er* and *FRI*-C24. The photoperiod was 16 hours, and Sylvania F58W/133 fluorescent tubes were used. Light intensity was in the range of 130 to 200 μE, and declined towards the sides of the cabinet. The racks were moved 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. The experiment was terminated after 100 days, as the growth medium in most tubes had dried out.

4.2.3 Analysis of F3 lines after measurement of flowering time

F3 plants from *fca*-AMT line #21 were genotyped for C24 and L*er* alleles at the *FRI* locus. DNA was isolated from single leaves or inflorescence tissue of individual plants from *fca*-AMT line #21 by the method described in Section 2.2.3, and the alleles of *FRI* present in 121 plants from this line were assessed using the *nga8* primers for PCR as described in Section 2.3.3. DNA for measurements of methyl-cytosine levels was extracted from pooled tissue for each line as described in Section 2.2.2. For *fca*-AMT line #21, DNA was extracted from 22 plants of which 4 plants were *FRI*-C24 homozygotes, 7 were *FRI*-L*er* homozygotes and 11 were heterozygous at the *FRI* locus. Methylation levels were assayed by a thin layer chromatography method described in Section 2.4.

4.3 Results

4.3.1 Selection of *METI* antisense-containing lines for the late flowering mutants

The F2 lines selected for measurement of flowering time were homozygous for the relevant late flowering mutant locus, and, in the case of lines derived from crosses with the homozygous *METI* antisense line T3#10.5 (AMT), contained the *METI* antisense transgene. The late flowering mutants and the *METI* antisense plants differed at the *FRI* locus, carrying *FRI*-L*er* and *FRI*-C24 alleles respectively. *FRI*-L*er* confers early flowering, while *FRI*-C24 confers late flowering, although in the presence of the "early" *FLC* allele carried by both L*er* and C24, this effect is small (Sanda and Amasino, 1995). The genotype of the F2 lines at the *FRI* locus was determined so that variations in flowering time due to *FRI* alleles could be avoided, or, at least, accounted for.

(a) *fe*

F2 plants, (*fe*-AMT), derived from crosses between *fe* and AMT were screened to indentify those that contained the *METI* antisense transgene. Of 15 plants screened, 13 contained the *Npt*II gene. The second stage of selection was to screen for homozygosity for the *fe* mutant allele. DNA from the 13 *fe*-AMT F2 plants, and from 9 *fe*-C24 F2 plants, was analysed by Southern blotting using the *AtADH* gene as an RFLP marker linked to *fe* (Chang and Meyerowitz, 1986; Dolferus *et al*, 1990). Four of the 13 *fe*-AMT F2 plants, and 2 of the 9 *fe*-C24 F2 plants, were homozygous for the L*er* allele of *AtADH*, and these were classed as being homozygous for the mutant *fe* allele.

The selected *fe*-AMT and *fe*-C24 F2 plants were screened to determine the alleles present at the *FRI* locus. All 4 of the *fe*-AMT F2 plants, #2, 7, 11 and 21, were homozygous for the C24 *nga8* allele linked to *FRI* (Bell and Ecker, 1994), and were classed as homozygotes for *FRI*-C24. One of these plants, #7, did not set seed. The progeny of plants #2 and #11 were used to measure flowering time. Of the 2 *fe*-C24 F2 plants, #3 was homozygous for the L*er* *nga8* allele, and #4 for the C24 *nga8* allele; these were classed as homozygotes for *FRI*-L*er* and *FRI*-C24 respectively. The flowering time of the progeny of *fe*-C24 #4 was used as the control for the *fe*-AMT lines, and the flowering times of the two *fe*-C24 lines were compared to investigate the effect of the *FRI* gene on flowering time. The genotypes of all the selected lines are shown in Table 4.1.

(b) *fca*

F2 plants, (*fca*-AMT), derived from crosses between *fca* and the *METI* antisense line T3#10.5 were screened to identify those which contained the *METI* antisense transgene. Of 23 plants screened, 21 contained the *Npt*II gene. These 21 F2 plants, and 14 *fca*-C24 F2 plants, were screened for homozygosity for the *fca* mutant allele by Southern analysis with the linked *CYP83* gene (Bilodeau *et al*., 1999). Only 1 of the 21 *fca*-AMT F2 plants, and 4 of the 14 *fca*-C24 F2 plants, were homozygous for the L*er* allele of *CYP83*, and these were classed as being homozygous for the mutant *fca* allele. It is unclear why only one *fca* homozygote was isolated from the cross to T3#10.5; normal Mendelian inheritance should have resulted in one quarter of the F2 plants being homozygous for *fca*. The chromosomal location of the *METI* antisense is unknown; if it has inserted into

Cross	F2 line	F2 *FRI* allele[1]
fe x T3#10.5	*fe*-AMT #2*	C24/C24[2]
fe x T3#10.5	*fe*-AMT #11*	C24/C24[2]
fe x C24	*fe*-C24 #3*	L*er*/L*er*[3]
fe x C24	*fe*-C24 #4*	C24/C24[2]
fca x T3#10.5	*fca*-AMT #21*	C24/L*er*
fca x C24	*fca*-C24 #1*	L*er*/L*er*[3]
fca x C24	*fca*-C24 #4	L*er*/L*er*[3]
fca x C24	*fca*-C24 #8	L*er*/L*er*[3]
fca x C24	*fca*-C24 #25	L*er*/L*er*[3]

Table 4.1: F2 lines generated from crosses of late flowering mutants *fe* and *fca* with *METI* antisense line T3#10.5 (antisense methyltransferase, AMT) and C24 wildtype. *Lines used in flowering time experiments; [1]*FRI* genotype based on the allele present at the linked nga8 locus; [2] *FRI*-C24 allele (confers late flowering); [3]*FRI*-L*er* allele (confers early flowering).

chromosome IV, which contains the *FCA* locus, this could account for the lower frequency of *METI* antisense-containing plants homozygous for the mutant *fca* allele.

The homozygous *fca*-AMT and *fca*-C24 F2 plants were screened for the alleles present at the *FRI* locus. The *fca*-AMT F2 plant, #21, was heterozygous at this locus. All 4 of the *fca*-C24 F2s, #1, 4, 8 and 25, were homozygous for the L*er* *nga8* allele, and were classed as homozygotes for *FRI*-L*er*. It is not surprising that no plants homozygous for the mutant *fca* allele from *Ler* and for *FRI*-C24 were identified, as the *FCA* and *FRI* loci are linked on chromosome 4.

The genotypes of the selected lines are shown in Table 4.1. The flowering time of the progeny of *fca*-AMT F2 #21 and *fca*-C24 F2 #1 were measured, as *fca*-C24 F2 #1 provided a control for the *FRI*-L*er* homozygotes in line *fca*-AMT F2 #21. Whereas 40 plants were grown for all other lines, for line *fca*-AMT #21, 200 plants were grown, and the *FRI*-L*er* homozygotes were identified after flowering by PCR for the *nga8* marker.

4.3.2 Vernalization response of late flowering mutant lines

The vernalization response of lines *fe*-C24 #4 and *fca*-C24 #1 was measured to determine whether any changes in vernalization responsiveness had occurred due to inheritance of alleles from the C24 parent. Observations of flowering time were made until 100 days after germination. Not all plants flowered in this time, so flowering time results will be

presented as percentages of plants flowering in 100 days, and time for 90% of plants to flower.

(a) *fe*

After 100 days, all 39 vernalized plants and 36 of 37 (97.3%) unvernalized plants from line *fe*-C24 #4 had flowered. The time for 90% of plants to flower was 29 days for unvernalized plants, and 20 days for vernalized plants. The range of flowering times for vernalized plants was 12 to 21 days, while for unvernalized plants, it was 16 to 32 days (Figure 4.5). This indicates that line *fe*-C24 #4 shows a small vernalization response, slightly larger than that found for *fe* by Koornneef *et al*. (1998b). The vernalization response of line *fe*-C24 #3 was much greater than that of line *fe*-C24 #4 or the *fe* parent, and this line will be considered separately in a later section.

(b) *fca*

For line *fca*-C24 #1, 33 of 36 (91.7%) unvernalized plants and 39 of 40 (97.5%) vernalized plants flowered in 100 days. The time for 90% of plants to flower was 89 days for unvernalized plants and 25 days for vernalized plants. Excluding those few plants which did not flower, the range of flowering times was 15 to 91 days for unvernalized plants, and 12 to 35 days for vernalized plants (Figure 4.6). A clear vernalization response is shown, as found for *fca* by Koornneef *et al*. (1998b); however the vernalization response seen for line *fca*-C24 #1 is greater than the response seen for *fca* by Koornneef *et al*. (1998b).

Figure 4.5: Days from germination to elongation of primary inflorescence for F3 lines from crosses between *fe* and either the T3 *METI* antisense line 10.5 (AMT) or C24. Genotypes of different *fe* lines are shown in Table 4.1. Flowering times for unvernalized (unvern) and vernalized (vern) plants are shown, and in each case the time for 60 % of plants to flower is indicated (▼); for lines in which more than 90% of plants flowered, the time for 90% of plants to flower is also shown (▼).

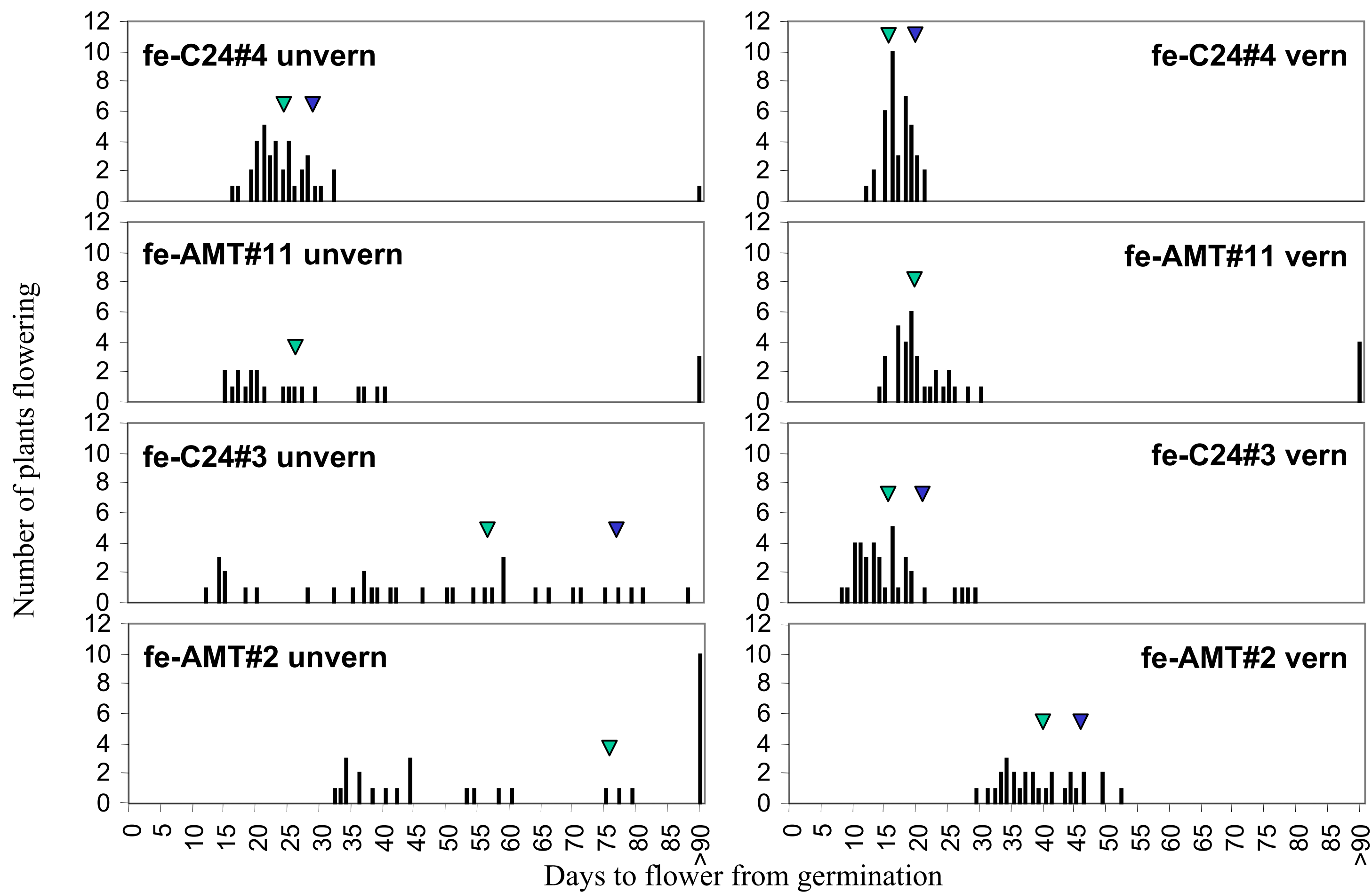
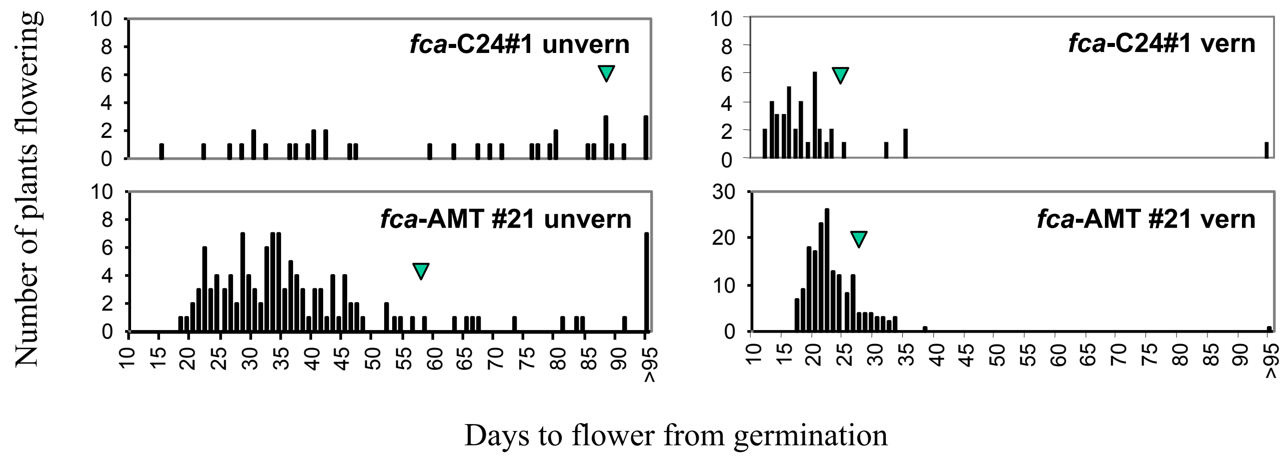

fe-C24#4 unvern
fe-C24#4 vern
fe-AMT#11 unvern
fe-AMT#11 vern
fe-C24#3 unvern
fe-C24#3 vern
fe-AMT#2 unvern
fe-AMT#2 vern
Number of plants flowering
Days to flower from germination
0
2
4
6
8
10
12
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
>90

Figure 4.6: Days from germination to elongation of primary inflorescence for F3 lines *fca*-AMT #21 and *fca*-C24 #1, from crosses between *fca* and either the T3 *METI* antisense line 10.5 (AMT) or C24. The genotypes of these lines are shown in Table 4.1. Flowering times for unvernalized (unvern) and vernalized (vern) plants are shown, and in each case the time for 90 % of plants to flower is indicated (▼). Note the larger scale for the graph showing flowering time of vernalized *fca*-AMT #21 plants; this is due to the larger number of plants grown for this line and the more clustered flowering times of the vernalized plants.


Number of plants flowering
fca-C24#1 unvern
fca-C24#1 vern
fca-AMT #21 unvern
fca-AMT #21 vern
Days to flower from germination

4.3.3 Demethylation caused by *METI* antisense expression

DNA was made from pooled tissue samples for each of lines *fe*-C24 #3, *fe*-C24 #4, *fe*-AMT #2, *fe*-AMT #11, *fca*-C24 #1 and *fca*-AMT #21, and DNA methylation levels were estimated. The pooled sample for line *fca*-AMT #21 was segregating at the *FRI* locus for L*er*-*FRI* and C24-*FRI*. Methylation levels for *fe*-AMT and *fca*-AMT lines were calculated relative to the methylation levels of control lines *fe*-C24 #4 and *fca*-C24 #1 respectively. DNA methylation was greatly decreased in both *fe*-AMT lines, with methylation in line *fe*-AMT #2 decreased to 35.7% of the control level, and methylation in line *fe*-AMT #11 decreased to 13.8% of the control level (Table 4.2). The level of DNA methylation for line *fca*-AMT #21 was decreased to 24.5% relative to that in line *fca*-C24 #1 (Table 4.2).

4.3.4 Effect of demethylation on flowering time of late flowering mutants

(a) *fe*

(i) lines *fe*-AMT #11 and *fe*-C24 #4

The time to flowering of vernalized and unvernalized F3 plants from lines *fe*-AMT #11 and *fe*-C24 #4 was compared. Both lines were homozygous for the C24 allele of the *FRI* gene. The experiment was terminated at 100 days. Not all plants flowered in this time, so flowering time data will be presented as percentages of plants flowering before 100 days, and time for 60% of plants to flower. Lines *fe*-AMT #2 and *fe*-C24 #3 were also grown

Line	5-methylcytosine
fca-C24 #1	100 %*
fca-AMT #21	24.5 %
fe-C24 #3	98.5 %
fe-C24 #4	100 %*
fe-AMT #2	35.7 %
fe-AMT #11	13.8 %

Table 4.2: 5-methylcytosine levels in F3 plants from crosses of late flowering mutants *fe* and *fca* with *METI* antisense line T3#10.5 (AMT), relative to the 5-methylcytosine levels of plants from crosses of late flowering mutants with C24. * Wildtype control lines.

in this experiment; the flowering behaviour of these lines differed from that of lines *fe*-AMT #11 and *fe*-C24 #4, and will therefore be discussed separately.

It should be noted that comparisons of flowering time between vernalized and unvernalized *fe*-C24 lines, and among *fca*-C24 and *fca*-AMT lines, were made using the time for 90% of plants to flower. As fewer plants in the *fe*-AMT lines flowered within 100 days, it is necessary to use the time for 60% of plants to flower to compare flowering of *fe*-C24 and *fe*-AMT lines.

After 100 days, over 97% of unvernalized *fe*-C24 #4 plants had flowered and 87% of unvernalized *fe*-AMT #11 plants had flowered (Table 4.3). The frequency distributions of flowering times for unvernalized plants from lines *fe*-AMT #11 and *fe*-C24 #4 are similar (Figure 4.5). In both lines, there appear to be two populations of plants: those which flowered, with flowering occurring from day 15 to day 40 for line *fe*-AMT #11, and day 16 to day 32 for *fe*-C24 #4; and the small number which did not flower in the 100 day period. The time for 60% of plants to flower was not significantly different between lines *fe*-AMT#11 and *fe*-C24 #4 (26 and 24 days respectively; Table 4.3). A small vernalization response was seen for line *fe*-C24 #4: unvernalized plants flowered from 16 to 32 days post-germination, and one plant failed to flower within 100 days; vernalized plants flowered from 12 to 21 days post-germination (Figure 4.5). Line *fe*-AMT #11 did not show a significant vernalization response (Figure 4.5). Demethylation caused by the introgression of the *METI* antisense construct into line *fe*-AMT #11 did not cause plants from this line to flower earlier than plants from line *fe*-C24 #4. This indicates that, in

Line	Treatment	% flowered (total no.)	Days for 90% to flower	Days for 60% to flower
fe-C24 #4 (*FRI*-C24)	unvernalized	97.3% (37)	29	24
	vernalized	100% (35)	20	16
fe-AMT #11 (*FRI*-C24)	unvernalized	87.0% (23)	n/a	26
	vernalized	88.9% (36)	n/a	20
fe-C24 #3 (*FRI*-L*er*)	unvernalized	100% (35)	77	56
	vernalized	100% (37)	21	16
fe-AMT #2 (*FRI*-C24)	unvernalized	66.7% (30)	n/a	75
	vernalized	100% (28)	46	40

Table 4.3: Flowering behaviour of unvernalized and vernalized F3 plants of four lines from crosses of *fe* with AMT or C24, showing percentage of plants that flowered in a 100 day period and the number of days for 90% or 60% of plants to flower.

plants which have little or no response to vernalization, demethylation does not promote flowering.

(ii) Lines *fe*-AMT #2 and *fe*-C24 #3

After 100 days, all unvernalized plants from line *fe*-C24 #3 and 67% of unvernalized plants from line *fe*-AMT #2 had flowered (Table 4.3). For both lines, unvernalized plants flowered later and over a longer period than unvernalized plants from lines *fe*-C24 #4 and *fe*-AMT #11 (Figure 4.5). The time for 60% of unvernalized plants to flower was 56 days for line *fe*-C24 #3, and 75 days for line *fe*-AMT #2. Both lines showed a large response to vernalization, unexpected for *fe* mutants. All unvernalized and vernalized plants for line *fe*-C24 #3 flowered within the 100 day period; flowering time ranged from 12 to 88 days for unvernalized plants, and from 8 to 29 days for vernalized plants (Figure 4.5). For line *fe*-AMT #2, 33% of unvernalized plants did not flower within 100 days, but all vernalized plants flowered within this period. Those unvernalized plants which flowered did so between day 32 and day 79. Vernalized plants flowered between day 29 and day 52 (Figure 4.5). Both the lateness of flowering and the restoration of the vernalization response in these two lines may be due to inheritance of C24-derived alleles of genes that regulate vernalization responsiveness and that differ between C24 and L*er*.

(b) *fca*

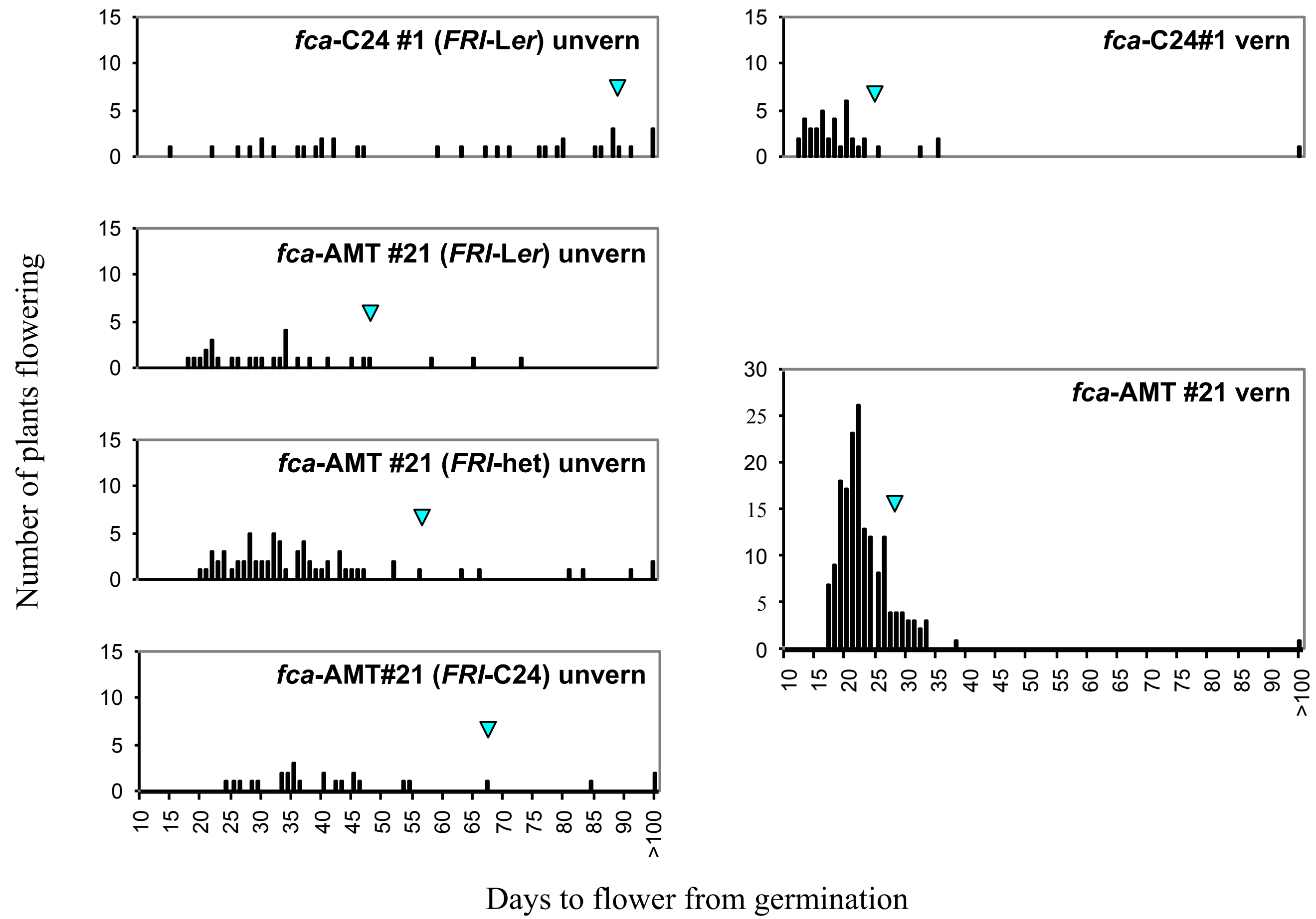
The flowering time of vernalized and unvernalized F3 plants from *fca*-AMT line #21 and *fca*-C24 line #1 was compared in order to assess the effect of demethylation on the flowering time of *fca*. F3 plants of *fca*-AMT line #21 were genotyped for the *FRI* locus using the linked *nga8* microsatellite marker. Of 121 unvernalized plants genotyped, 29 were homozygous for the L*er* allele of *FRI*, 66 were heterozygous at this locus, and 26 were homozygous for the C24 allele of *FRI*, consistent with the expected 1:2:1 ratio. The experiment was terminated at 100 days; as not all plants flowered in this time, flowering time results are presented as percentages of plants flowering before 100 days, and time for 90% of plants to flower.

Like the control line *fca*-C24 #1, plants from line *fca*-AMT #21 showed a vernalization response, as expected for *fca* (Table 4.4, Figure 4.6). Flowering of line *fca*-AMT #21 was strongly promoted relative to line *fca*-C24 #1: 90% of *fca*-AMT #21 plants flowered within 58 days, whereas it took 89 days for 90% of *fca*-C24 #1 plants to flower (Table 4.4, Figure 4.6). This promotive effect of demethylation in the *fca* background was seen regardless of *FRI* genotype. For line *fca*-AMT #21, the period for 90% of plants to flower was 48 days for *FRI*-L*er* homozygotes, 56 days for plants heterozygous at *FRI*, and 67 days for *FRI*-C24 homozygotes (Table 4.4, Figure 4.7).

Line	Treatment	% flowered (total no.)	Days for 90% to flower
fca-C24 #1 (*FRI*-L*er*)	unvernalized	91.7% (36)	89
	vernalized	97.5% (40)	25
fca-AMT #21 (all plants)	unvernalized	94.4% (124)	58
	vernalized	99.4% (170)	28
fca-AMT #21 (*FRI*-L*er*)	unvernalized	100 % (29)	48
fca-AMT #21 (*FRI*-L*er*/C24)	unvernalized	97.0 % (66)	56
fca-AMT #21 (*FRI*-C24)	unvernalized	92.3 % (26)	67

Table 4.4: Flowering behaviour of unvernalized and vernalized F3 plants of two lines from crosses of *fca* with AMT or C24, showing percentage of plants that flowered in a 100 day period and the number of days for 90% of plants to flower. Segregants for the *FRI* locus in line *fca*-AMT #21 are also shown separately.

Figure 4.7: Days from germination to elongation of primary inflorescence for F3 lines *fca*-AMT #21 and *fca*-C24 #1. Flowering times for unvernalized (unvern) and vernalized (vern) plants are shown, and in each case the time for 90 % of plants to flower is indicated (▽). Flowering time is presented separately for different *FRI* genotype classes of unvernalized plants from line *fca*-AMT #21.



fca-C24 #1 (FRI-Ler) unvern
fca-C24#1 vern
fca-AMT #21 (FRI-Ler) unvern
fca-AMT #21 (FRI-het) unvern
fca-AMT#21 (FRI-C24) unvern
fca-AMT #21 vern
Number of plants flowering
Days to flower from germination
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
>100

4.3.5 Effect of *FRI* on flowering time

The effect on flowering time of the different alleles of *FRI* inherited from the *METI* antisense plant (C24-*FRI*) or the late flowering mutants (L*er*-*FRI*) was examined. Where possible the interaction of decreased DNA methylation and *FRI* allelism was also measured.

(a) *fe*

Line *fe*-C24 #4 was homozygous for the C24 *FRI* allele (and was therefore the control line for the two demethylated *fe* lines) while line *fe*-C24 #3 carried the L*er* *FRI* allele. Paradoxically, line #3, carrying the early flowering *FRI* allele, flowered later than line #4, which carried the late flowering *FRI* allele (Table 4.3, Figure 4.5). Also, line #3 showed a large vernalization response, while line #4 had a much smaller vernalization response (Figure 4.5). As discussed above, it is likely that a locus (loci) that regulates flowering time and that differs between C24 and L*er* has been differentially inherited by these lines.

(b) *fca*

As the *fca*-AMT line #21 was segregating at the *FRI* locus, it was possible to compare the effect of flowering time of F3 plants homozygous for either C24 or L*er* *FRI*. These comparisons were made using data for the first 90% of plants to flower. Plants homozygous for C24 *FRI* flowered significantly later than those carrying L*er* *FRI* (Table

4.4, Figure 4.7). The flowering time for plants which were heterozygous at the *FRI* locus was intermediate to the two homozygote types, but there was no statistically significant difference between the flowering time of the heterozygotes compared to either homozygote. This indicates that in plants homozygous for *fca*, the *FRI*-C24 allele delays flowering in a dose-dependent manner.

4.4 Discussion

The results reported in Chapter 3 demonstrated the involvement of cytosine methylation changes in the regulation of flowering time in Arabidopsis. C24 and Columbia plants, in which DNA was demethylated by expression of an antisense construct to the methyltransferase gene *METI* (Finnegan and Dennis, 1993; Finnegan *et al*., 1996) or by mutation of the *DDM1* gene (decreased DNA methylation; Vongs *et al*., 1993), flowered early in conditions which allowed a vernalization response. These results suggested that demethylation promotes flowering by the vernalization pathway.

To study this correlation further, the effect of *METI* antisense-induced demethylation on flowering time was tested in two late flowering mutants which differ in vernalization responsiveness. The hypothesis predicts that, if demethylation causes early flowering via the vernalization pathway, demethylation should promote flowering in vernalization responsive plants, while not affecting flowering time in a vernalization unresponsive background. This was found to be the case when demethylation was effected using the demethylating agent 5-aza-C (Burn *et al.*, 1993a). The vernalization responsive crucifer

Thlaspi arvense, and vernalization responsive late flowering mutants of Arabidopsis, flowered early when treated with 5-aza-C, whereas the flowering of vernalization unresponsive late flowering mutants of Arabidopsis was not promoted.

The observed effects of demethylation in the vernalization responsive *fca* mutant background were consistent with the hypothesis that demethylation promotes flowering via the vernalization pathway. Both the *METI* antisense-containing *fca* line and the control line showed a large response to vernalization, and unvernalized plants from the *METI* antisense line flowered earlier than unvernalized control plants.

Two categories of *fe* lines were seen, based on flowering time and vernalization responsiveness. Lines *fe*-C24 #4 and *fe*-AMT #11 had small vernalization responses, and no early flowering response to demethylation was seen. Lines *fe*-C24 #3 and *fe*-AMT #2 both showed large responses to vernalization, and both flowered late relative to lines *fe*-C24 #4 and *fe*-AMT #11. These results were unexpected, based on the known genotypes of these lines; all four lines were homozygous for the mutant *fe* allele, and all lines but *fe*-C24 #3 were homozygous for the C24 allele of *FRI*, which confers late flowering (Sanda and Amasino, 1995). Line *fe*-C24 #3 was homozygous for the early flowering L*er* allele of *FRI* (Koornneef *et al*., 1994), and was therefore expected to flower earlier than *fe*-C24 #4. As lines *fe*-AMT #2 and *fe*-AMT #11 had the same genotype at the *FE* and *FRI* loci, they were expected to have similar flowering times and responses to vernalization and demethylation. The observed differences in flowering time between these two sets of lines indicate that a locus or loci other than *FE* and *FRI* are influencing flowering time,

and it is most likely that a locus or loci inherited from C24 caused the late flowering and vernalization responsiveness in lines *fe*-C24 #3 and *fe*-AMT #2. These observations are consistent with the finding that F2 plants derived from a cross between C24 and L*er*, which did not inherit the late *FRI* allele from C24, segregated for late flowering, demonstrating that another locus (or loci) which regulates flowering differs between these ecotypes (C.C. Sheldon, personal communication).

Sheldon *et al*. (1999) showed that expression of the flowering repressor *FLC* (*FLF*) is up-regulated in late flowering mutants from the vernalization responsive group, including *fca*, but not in late flowering mutants such as *fe*, which are less responsive, or not responsive, to vernalization (Koornneef *et al*., 1998b). Also, *FLC* expression is down-regulated in vernalized plants and *METI* antisense transgenics (Sheldon *et al*., 1999; Michaels and Amasino, 1999). The level of *FLC* mRNA was measured for the *fe* lines by Northern analysis (E.J. Finnegan, personal communication). *FLC* mRNA was undetectable for line *fe*-AMT #11, which showed little vernalization response, while vernalization responsive line *fe*-AMT #2 had a high level of *FLC* mRNA. A similar pattern was seen for the two *fe*-C24 lines: line *fe*-C24 #4 had a very low level of *FLC* expression and a small vernalization response, while line *fe*-C24 #3 had a high level of *FLC* expression and responded strongly to vernalization (Table 4.3; E.J. Finnegan, personal communication). *FLC* expression is much higher in C24 than in L*er* (Sheldon *et al*., 1999), so the increase in *FLC* expression in lines *fe*-AMT #2 and *fe*-C24 #3 is likely to be due to inheritance of a locus from C24 which increases *FLC*. Plants with increased *FLC* expression flower late and respond to vernalization (Sheldon *et al*., 1999; Michaels

and Amasino, 1999), so the increased *FLC* levels in lines *fe*-AMT #2 and *fe*-C24 #3 suggest a mechanism for the observed late flowering, vernalization responsiveness and promotion of flowering by demethylation.

FLC is expressed at a higher level in *fca* than in wildtype L*er*, suggesting that wildtype FCA may down-regulate *FLC* expression (Sheldon *et al*., 1999). Levels of *FLC* are reduced in *fca*-AMT #21 relative to *fca*-C24 #1 (E.J. Finnegan, personal communication); this is likely to be due to demethylation by *METI* antisense, which reduces *FLC* expression (Sheldon *et al*., 1999). These results suggest that expression of *METI* antisense in the *fca* background promoted flowering through down-regulation of *FLC* expression. The late flowering phenotype of *fe*, which is not due to increased *FLC* expression, was not reversed by *METI* antisense expression. This suggests that the promotion of flowering by demethylation may act specifically through regulating *FLC* expression.

According to the hypothesis that demethylation substitutes for the vernalization response, the vernalization responsive line *fe*-AMT #2 might have been expected to flower early in response to demethylation. Line *fe*-AMT #2 may have flowered early relative to a perfect control line - that is, a control line genotypically identical to line #2 except for the absence of the *METI* antisense. The lack of a perfect wildtype control for the antisense-containing lines could be overcome by repeated backcrossing to the late flowering mutant, in order to produce near isogenic lines. Direct transformation of the late

flowering mutants with the *METI* antisense construct, which was attempted unsuccessfully in this study, would also avoid this problem.

Line *fca*-AMT #21 was segregating for the *FRI* locus, which allowed the effect of *FRI* on flowering time to be examined in this line. Plants homozygous for the late allele, *FRI*-C24, flowered later than those homozygous for the early allele, *FRI*-L*er*. The flowering time of plants heterozygous at this locus was intermediate to the flowering time of the two homozygotes, in contrast to the findings of Lee *et al*. (1993, 1994a). When the late flowering *FRI* allele of ecotype San Feliu-2 (*FRI*-Sf2) was introgressed into the L*er* background, although the late flowering phenotype was partially suppressed in this background, *FRI*-Sf2 was fully dominant. Results reported here suggest that the late allele *FRI*-C24 is semi-dominant. Introgression of *FRI*-C24 into the L*er* background and measurement of the flowering time of homozygotes and heterozygotes for *FRI*-C24 would determine whether the *FRI*-C24 allele is dominant or semi-dominant.

In conclusion, the results reported here support the hypothesis that the promotive effect of demethylation on flowering time operates through the vernalization pathway. Lines *fca*-C24 #1 and *fca*-AMT #21 were strongly vernalization responsive, and *fca*-AMT #21, which had substantial DNA demethylation, flowered earlier than *fca*-C24 #1. Lines *fe*-C24 #4 and *fe*-AMT #11 had small or non-existent vernalization responses, and flowering time was not promoted in the AMT line. The results reported for expression levels of the floral repressor *FLF* (E.J. Finnegan, personal communication) support the observations by Sheldon *et al*. (1999) that *FLF* levels are reduced in *METI* antisense plants, and

suggest that the mechanism by which demethylation promotes flowering is down-regulation of *FLF* expression.

The 2 classes of flowering time behaviour observed for different *fe* lines indicates that a locus or loci regulating flowering time, probably via regulation of *FLC* expression, was differentially inherited from C24. These observations have uncovered another gene(s) involved in regulation of flowering time which differs between L*er* and C24.

In the studies reported here, the AMT transgene was introduced into the *fca* and *fe* mutants by crossing. Because the background ecotypes of the AMT transgenics and the late flowering mutants were different, segregation of other flowering time genes complicated the analysis. A clearer picture of the effect of demethylation on the flowering of late flowering mutants that differ in vernalization responsiveness may be gained by direct transformation of these mutants with the AMT transgene. Generation of AMT transgenics has been successful for a number of late flowering mutants (E.J. Finnegan, personal communication), and the effect of demethylation on flowering time of these mutants will be observed.