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The downregulation of *FLOWERING LOCUS C* (*FLC*) expression in plants with low levels of DNA methylation and by vernalization occurs by distinct mechanisms

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Summary

FLOWERING LOCUS C (FLC), a repressor of flowering, is a major determinant of flowering time in Arabidopsis. *FLC* expression is repressed by vernalization and in plants with low levels of DNA methylation, resulting in early flowering. This repression is not associated with changes of DNA methylation within the *FLC* locus in either vernalized plants or plants with low levels of DNA methylation. In both cases, there is a reduction of histone H3 trimethyl-lysine 4 (K4) and acetylation of both histones H3 and H4 around the promoter-translation start of *FLC*. The expression of the two genes flanking *FLC* is also repressed in both conditions and repression is associated with decreased histone H3 acetylation. The changes in histone modifications at the *FLC* gene cluster, which are similar in vernalized plants and in plants with reduced DNA methylation, must arise by different mechanisms. VERNALIZATION 1, VERNALIZATION 2 and VERNALIZATION INSENSITIVE 3 modulate *FLC* expression in vernalized plants; these proteins play no role in the downregulation of *FLC* in plants with low levels of DNA methylation. Chimeric *FLC::GUS* transgenes respond to vernalization but these same transgenes show a position-dependent response to low levels of DNA methylation. In plants with reduced DNA methylation, expression of the five *MADS AFFECTING FLOWERING* (*MAF*) genes is repressed, suggesting that DNA methylation alters the expression of a trans-acting regulator common to *FLC* and members of the related *MAF* gene family. Our observations suggest that DNA methylation is not part of the vernalization pathway.

Keywords: vernalization, DNA methylation, histone acetylation, chromatin immunoprecipitation, *VRN1*, *VIN3*.

Introduction

Flowering in Arabidopsis can be induced by two major environmental cues, photoperiod and low temperature. In the absence of either of these cues, flowering will occur as a consequence of the developmental or autonomous pathway (Boss *et al.*, 2004). *FLOWERING LOCUS C (FLC)*, which encodes a repressor of flowering, is a key gene in both the autonomous and vernalization pathways (Sheldon *et al.*, 1999, 2000). Regulation of *FLC* is multifactorial and involves both genetic and epigenetic mechanisms including histone modification and DNA methylation. *FLC* expression is upregulated by FRIGIDA (FRI) and two related proteins, FRIGIDA LIKE 1 (FRL1) and FRL2 (Figure 1; Johanson *et al.*, 2000; Michaels and Amasino, 1999; Michaels *et al.*, 2004; Sheldon *et al.*, 1999). Genes of the autonomous pathway, such as *FCA, FVE* and *FLD*, act as negative regulators of *FLC* (Figure 1); when these genes are mutated, the level of *FLC* expression is elevated, even in plants lacking active alleles of *FRI* (Sheldon *et al.*, 1999).

The upregulation of *FLC* by genes of the *FRI* family and in autonomous pathway mutants is dependent on the activity of a number of other genes, some of which have been shown to affect the structure of chromatin at the *FLC* locus. Arabidopsis homologues of the budding yeast RNA polymerase (Pol II) Associated Factor 1 (Paf1) complex, VERNALIZATION INDEPENDENT 4 (VIP4), VIP5, EARLY FLOWERING 7 (ELF7) and VIP6/ELF8 are essential for the promotion of *FLC* expression by FRI and in autonomous pathway mutants (Figure 1; He *et al.*, 2004; Oh *et al.*,

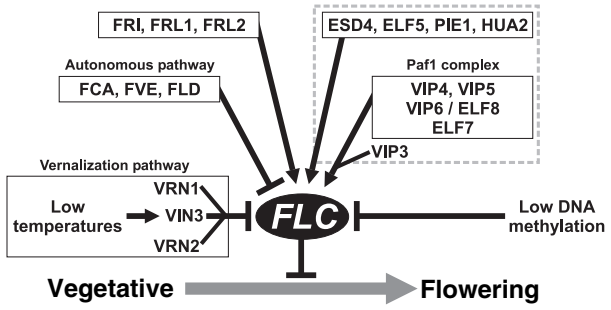

Figure 1. The regulation of *FLOWERING LOCUS C* (*FLC*) involves a complex network of pathways.
FLC expression is upregulated by genes of the *FRI* clade and when genes in the autonomous flowering pathway are mutated. The activation of *FLC* expression by the FRIGIDA (FRI) gene family and by autonomous pathway mutants is dependent on the activity of proteins of the Paf1 complex and other proteins of unknown function (indicated by box enclosed by the dashed grey line). As demonstrated in this paper, the downregulation of *FLC* by vernalization and by low DNA methylation occurs by two distinct pathways.

2004; Zhang and van Knocker, 2002). VIP3, which has no counterpart in budding yeast, interacts with VIP6 and so may be part of this complex in Arabidopsis (Oh *et al.*, 2004; Zhang *et al.*, 2003). In yeast, components of the Paf1 complex associate with the initiating and elongating forms of RNA polymerase II (Mueller and Jaehning, 2002) and are localized within the 5′ regions of some genes (Krogan *et al.*, 2002). Mutants of the Arabidopsis Paf1 complex show reduced expression not only of *FLC*, but also of the related *MADS AFFECTING FLOWERING* (*MAF*) gene family (He *et al.*, 2004).

The importance of chromatin structure in the activation of *FLC* is also demonstrated by the finding that PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE1), an Imitation Switch (ISWI) chromatin remodelling protein, is essential for the activation of *FLC* expression in plants carrying active alleles of FRI and in autonomous pathway mutants (Noh and Amasino, 2003). Several other genes, including *ELF5*, *EARLY IN SHORT DAYS 4* (*ESD4*) and *HUA2*, are also required for *FLC* expression in both autonomous pathway mutants and plants with an active FRI (Figure 1; Doyle *et al.*, 2005; Noh *et al.*, 2004; Reeves *et al.*, 2002).

Flowering is promoted by prolonged exposure to low temperatures (vernalization). Vernalization represses *FLC* expression, over-riding the positive effects of FRI or mutants in the autonomous pathway. The downregulation of *FLC* in vernalized plants is associated with a decrease in histone H3 acetylation and increased methylation of lysines 9 and 27 (Bastow *et al.*, 2004; Sung and Amasino, 2004). Several genes in the vernalization pathway have been identified (Figure 1). *VERNALIZATION INSENSITIVE 3* (*VIN3*) is induced in response to prolonged cold and its activity is essential for the downregulation of *FLC* and the associated decrease in histone H3 acetylation (Sung and Amasino, 2004). VERNALIZATION 1 (VRN1) and VRN2 are required for the complete repression of *FLC* in vernalized plants and for methylation of lysine 9 or lysines 9 and 27, respectively (Bastow *et al.*, 2004; Sung and Amasino, 2004).

Changes in DNA methylation affect chromatin structure and *vice versa*, suggesting that chromatin structure is intimately linked to the methylation status of DNA. Methylation of DNA has been associated with deacetylation of histone H3 and with methylation of histone H3 at lysine 9 (Gendrel *et al.*, 2002; Ng and Bird, 1999). DNA methyltransferases have been found in protein complexes containing histone deacetylase or histone methyltransferase activity (Fuks *et al.*, 2003; Rountree *et al.*, 2000). Conversely, H3 lysine 9 methylation is essential for methylation of associated DNA under some circumstances (Jackson *et al.*, 2002; Tamaru and Selker, 2001). The link between DNA methylation and chromatin structure is reinforced by the finding that mutation of a chromatin remodelling protein, DECREASED DNA METHYLATION 1 (DDM1), results in loss of DNA methylation (Brzeski and Jerzmanowski, 2003; Jeddeloh *et al.*, 1999; Vongs *et al.*, 1993). The close connection between these modifications raises the possibility that DNA methylation may play an integral role in the vernalization response.

Two lines of evidence suggest that DNA methylation is involved in the vernalization response. Vernalization is associated with a small, transient decrease in DNA methylation (Burn *et al.*, 1993; Finnegan *et al.*, 1998) and *FLC* is downregulated in plants where the level of DNA methylation has been reduced by an antisense construct against the DNA methyltransferase, *METHYLTRANSFERASE I* (*METI*; Sheldon *et al.*, 1999). In both vernalized plants and plants with low levels of DNA methylation, the genes flanking *FLC* are downregulated along with *FLC*. The coordinated response of these three adjacent genes supports the premise that changes in chromatin across a region of about 12 kb affect the expression of these genes (Finnegan *et al.*, 2004).

In this paper, we compare the downregulation of *FLC* induced by vernalization and in plants with low levels of DNA methylation. We find that both vernalization and demethylation of DNA are associated with changes in histone modification at *FLC* and its two flanking genes. There is a reduction of both histone H3 acetylation and H3 trimethyl-K4 at each gene in the cluster, whereas histone H4 acetylation is decreased only at *FLC*. *FLC* is not directly regulated by DNA methylation as there is no change in DNA methylation within the *FLC* gene segment that is necessary for the vernalization response (Sheldon *et al.*, 2002). Genes that are known to mediate the repression of *FLC* in vernalized plants play no role in the low methylation-induced repression of *FLC*. Although there are similarities between the response of *FLC* to vernalization and to low DNA methylation, these two pathways inducing the chromatin-based epigenetic control of *FLC* activity appear to be distinct.

Results

FLC *is downregulated in plants with low levels of CpG methylation but not in plants with reduced levels of CpNpG methylation*

METI antisense (*AMT*) plants have reduced levels of CpG methylation but little change in methylation of cytosines located in either CpNpG or asymmetric sequences (Kishimoto *et al.*, 2001). In C24 plants with about 20% of the normal level of CpG methylation, the time to flowering decreased from 87 to 47 days and *FLC* transcript levels were reduced by 80–90% (Finnegan *et al.*, 1998; Sheldon *et al.*, 1999). To determine whether loss of DNA methylation in sequence contexts other than CpG dinucleotides affects *FLC* expression, we generated plants with reduced levels of CpNpG and asymmetric methylation using RNAinterference (RNAi) against *CHROMOMETHYLASE3* (*CMT3*), which plays a role in the maintenance of methylation of cytosines located in non-CpG sequence contexts (Bartee *et al.*, 2001; Cao *et al.*, 2003; Lindroth *et al.*, 2001; Smith *et al.*, 2000). We selected plants with low *CMT3* activity by assaying the level of methylation of CpCpG sites in centromeric repeats (Figure 2a). We found no changes in either flowering time or *FLC* expression in the plants with reduced CpNpG methylation (Figure 2b,c). Furthermore, reduced *CMT3* activity did not affect the downregulation of *FLC* by vernalization (Figure 2b,c).

Mutation of the *DDM1* gene reduces cytosine methylation in all sequence contexts by an unknown mechanism (Vongs *et al.*, 1993). We found that *FLC* was downregulated in the *ddm1-1* mutant, just as in *AMT* plants (Figure 2d). Taken together, the data from *AMT*, *CMT3*-RNAi and *ddm1* plants suggest that *FLC* expression is influenced by the genomic level of CpG methylation, but not by the level of methylation of cytosines in other sequence contexts.

The repression of FLC *by vernalization or by a genome-wide reduction in DNA methylation is not mediated by changes in DNA methylation at the* FLC *locus*

As changes in gene expression are often associated with changes in DNA methylation within the regulatory region of a gene, we examined the methylation status of cytosines within the region of *FLC* (bases −2029 to +3703; Figure 3a) that confers a vernalization response on a reporter gene (Sheldon *et al.*, 2002). Southern analyses using methylation sensitive enzymes or McrBC, an enzyme that cleaves methylated DNA in a sequence-independent manner (Sutherland *et al.*, 1992), show that this region of the gene is not substantially methylated in control plants and that there is no change in methylation of this region in vernalized plants (Figure 3b). Of 956 cytosines sampled by bisulphite sequencing of the coding strand, 111 of which were located in CpG dinucleotides, only one cytosine was found to be methylated. This is located in a CpG dinucleotide within intron 1, 2360 bases downstream of the translation start; the corresponding cytosine on the non-coding strand is also

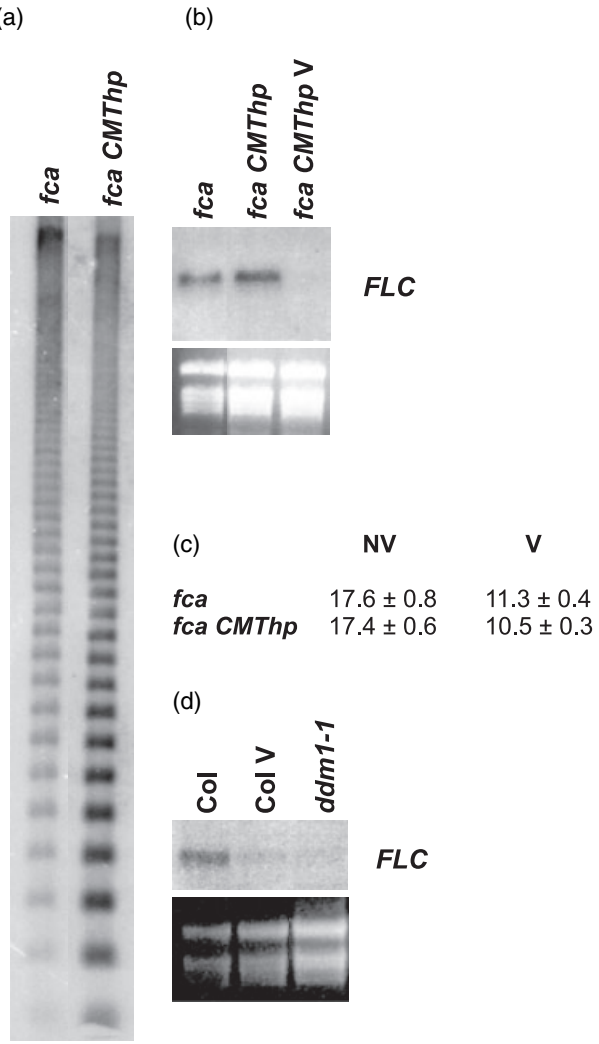

Figure 2. Reducing CpNpG methylation does not affect *FLOWERING LOCUS C* (*FLC*) expression or the vernalization response.
(a) Plants homozygous for the *fca-1* mutation were transformed with a hairpin silencing construct targeting *CHROMOMETHYLASE3* (*CMT; CMT3hp*). Plants with reduced CMT3 activity leading to a reduction in CpNpG methylation were identified by Southern analysis of DNA digested with *Msp*I and probed with the centromeric repeat fragment (Martinez-Zapater *et al.*, 1986). Equivalent amounts of DNA were loaded for each sample as judged from the ethidium bromide stained gel.
(b) Northern analysis of RNA isolated from plants with normal or reduced (*CMT3hp*) levels of CpNpG methylation showing that there was no difference in *FLC* expression in non-vernalized (NV) plants and that *FLC* was repressed by vernalization in these plants.
(c) Rosette leaf number at flowering for *fca-1* and *fca-1 CMT3hp* plants showing that the *CMT3hp* had no effect on the flowering time of either NV or vernalized (V) plants.
(d) Northern analysis of RNA isolated from Columbia (Col) and *ddm1-1* showing that *FLC* expression is repressed in the *decreased DNA methylation 1-1* (*ddm1-1*) mutant with low levels of DNA methylation.

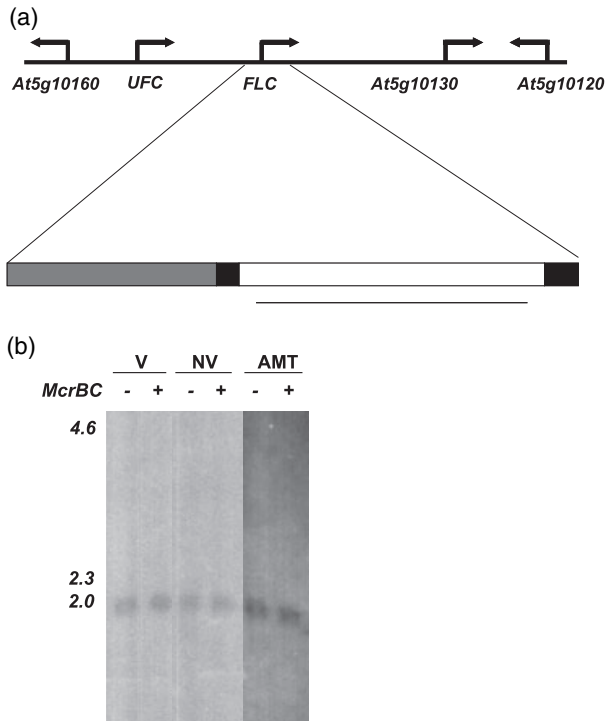

Figure 3. The downregulation of *FLOWERING LOCUS C* (*FLC*) is not associated with changes in DNA methylation of the *FLC* locus.
(a) Diagram of the genomic region surrounding *FLC*. The bar underneath the map indicates the region of the *FLC* gene that was analysed by bisulphite sequencing; the grey bar represents the promoter (2 kb), the solid bars represent exons 1 and 2 and the white bar represents intron 1 (3.5 kb). The same region was fused to the β-*GLUCURONIDASE* (*GUS*) gene in the 6 kb-*FLC::GUS* reporter construct. The line below the white bar indicates the region of the intron deleted in the Δ2.8 kb-*FLC::GUS* construct.
(b) Southern analysis showing that the *FLC* promoter (grey bar in panel a) was not methylated in non-vernalized (NV) C24 plants, in vernalized (V) plants or in plants with reduced levels of CpG methylation (METI antisense). The DNA was digested with the two enzymes *Bam*HI and *Nco*I (–) or with the same two enzymes plus McrBC (+), as indicated above the blot. Probing with the intron sequences gave an identical result (not shown).

methylated (Table S1). The methylation status of this site must not be important in regulating *FLC* transcription as it is methylated in both vernalized and control plants (Table S1). This analysis showed that there are no vernalization-induced changes in DNA methylation over the 6.1-kb region of the *FLC* gene examined.

In *AMT* plants, where the level of genome methylation is substantially lower than that in control plants, bisulphite sequencing showed that neither cytosine$_{2360}$ nor the corresponding cytosine on the non-coding strand was methylated (Table S1). There were no other changes in DNA methylation in the *FLC* gene in these plants (Figure 3b). Therefore, changes in DNA methylation of the *FLC* gene itself can not be responsible for repression of this gene by either vernalization or by low levels of genomic DNA methylation (see below).

The downregulation of FLC *is associated with histone deacetylation and a reduction in histone H3 trimethyl-lysine 4 in both vernalized plants and in plants with low levels of DNA methylation*

Vernalization is associated with a decrease in histone H3 acetylation at the promoter and first intron of the *FLC* locus (Sung and Amasino, 2004). We have used chromatin immunoprecipitation (ChIP) assays to assess the level of acetylation of histones H3 and H4 associated with the promoter-translation start of the *FLC* gene in both vernalized and *AMT* plants. In vernalized plants harvested at the end of the cold treatment, the level of histone H3 acetylation decreased to approximately 70% of that seen in non-vernalized controls (Figure 4a). The level of H3 acetylation associated with *FLC* remained low when plants were transferred to warmer conditions following the cold treatment (Figure 4a); in contrast to earlier reports, we saw no decrease in H3 acetylation during this period (Sung and Amasino, 2004). When *FLC* expression was repressed by reduced DNA methylation rather than by vernalization, the level of histone H3 acetylation was again decreased, in this case to about 50–60% of the control level (Figure 4a).

Histone H4 acetylation at the *FLC* gene was also decreased in both vernalized and *AMT* plants (Figure 4a). While histone H3 acetylation was reduced across the translation start of *FLC* (−201 to +8) as well as in a region upstream of the transcription start (−722 to −514), the decrease in histone H4 acetylation did not extend into the region upstream of the transcription start (Figure S1). The reduction in H4 acetylation at *FLC* was stable during the period following the cold treatment.

Trimethylation of K4 in histone H3, which is localized to the 5′ region of a gene, is a mark of transcriptional activity (Santos-Rosa *et al.*, 2002). In non-vernalized plants, we found H3 trimethyl-K4 associated with the translation start of *FLC* (−201 to +8), but not in the region upstream of the transcription start (Figure 4b; not shown). In both vernalized plants and in plants with low DNA methylation, the level of H3 trimethyl-K4 at *FLC* decreased (Figure 4b). A reduction in histone acetylation and H3 trimethyl-K4 at *FLC* is a common feature of both vernalization and low levels of DNA methylation.

Downregulation of FLC *in plants with low DNA methylation is not associated with activation of* VIN3

As neither vernalization-induced nor low methylation-induced repression of *FLC* is mediated by changes in DNA methylation of the *FLC* gene itself, DNA methylation may control the expression of a trans-acting repressor of *FLC*. We considered the possibility that *VIN3*, which is essential for the establishment of *FLC* repression and for the loss of histone acetylation around the *FLC* gene in V plants (Sung and

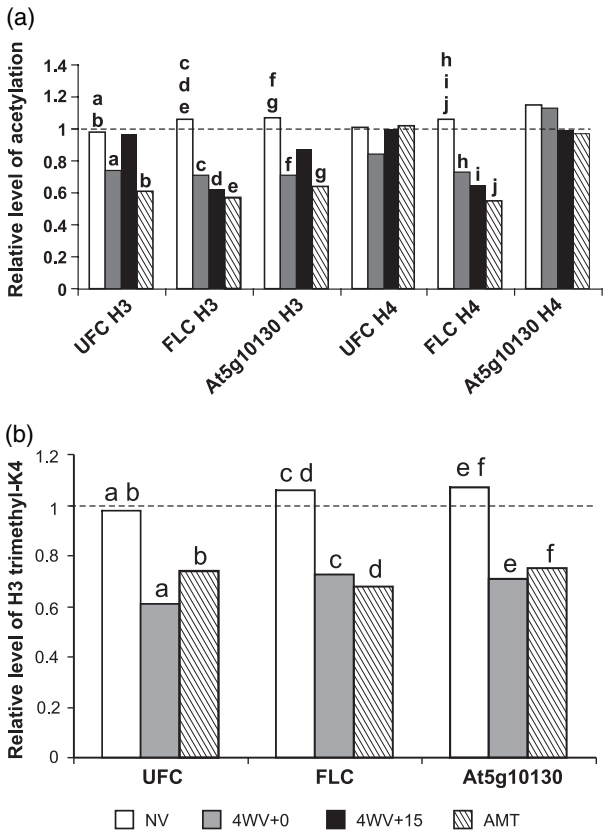

Figure 4. The level of histone acetylation and H3 trimethyl-K4 at genes within the *FLOWERING LOCUS C* (*FLC*) cluster is reduced in vernalized (V) plants and in plants with low levels of DNA methylation.

(a) The histograms represent the level of histone H3 and H4 acetylation, determined by chromatin immunoprecipitation (ChIP), in control 15-day-old plants (non-vernalized, NV), plants harvested at the end of a 4-week vernalization (4WV + 0), plants harvested 15 days after the end of a 4-week vernalization (4WV + 15) and *AMT* plants (AMT) compared to control 15-day-old plants.

(b) The histograms represent the level of histone H3 trimethyl-K4, determined by ChIP, in control 15-day-old plants (NV), plants harvested at the end of a 4-week vernalization (4WV + 0) and *AMT* plants (AMT) compared to control 15-day-old plants.

Histograms marked with the same letter indicate differences that are significant ($P < 0.05$; for panel b: b, $P = 0.05$). The data for this figure were obtained using the primer pairs UFC (−238 to +90), FLC2 (−201 to +8) and At5g10130 (−88 to +118); coordinates are given relative to the translation start. The control gene for all treatments was *S-ADENOSYL METHIONINE SYNTHASE* (*SAM*; At4g01850) whose expression does not change following vernalization (Finnegan *et al.*, 2004) or in plants with low levels of DNA methylation. For *UFC* and *At5g10130*, the upstream primer pairs gave similar results (data not shown), whereas for *FLC* there was no reduction in histone H4 acetylation in the upstream region (Figure S1). The data presented are the average values for at least three experiments.

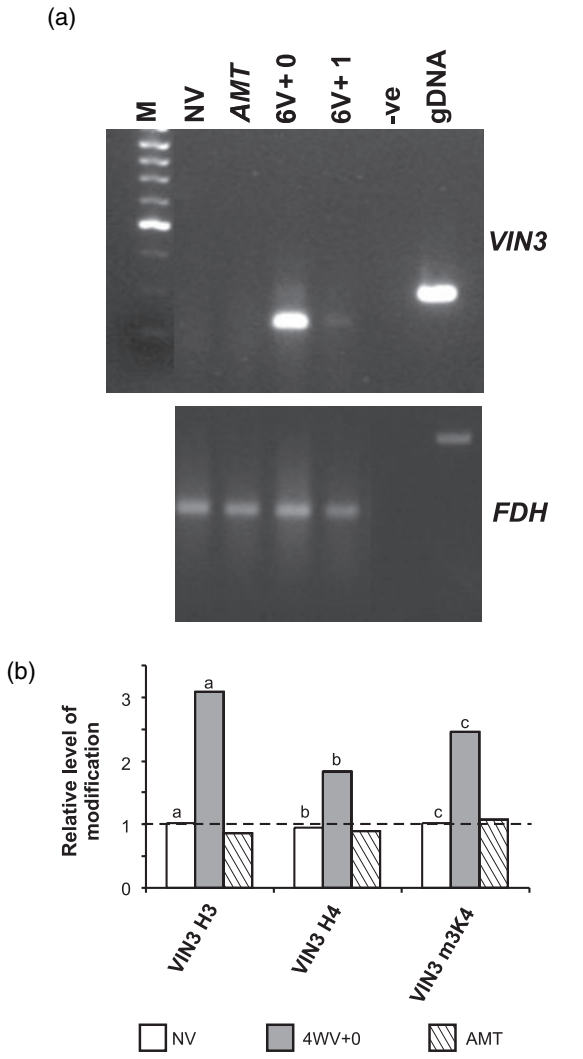

Figure 5. The downregulation of *FLOWERING LOCUS C* (*FLC*) in plants with low levels of DNA methylation is not associated with induction of *VERNALIZATION INSENSITIVE 3* (*VIN3*) expression.

(a) Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *VIN3* expression in control plants (non-vernalized, NV), plants with low levels of DNA methylation (*AMT*) and plants harvested at the end of a 6-week vernalization (6V +0) or 1 day after the end of the cold treatment (6V +1). No template (−ve) and genomic DNA (gDNA) controls are indicated. A control RT-PCR using primers for *FORMALDEHYDE DEHYDROGENASE* (*FDH*, *At5g43940*) is shown in the lower panel to quantify input RNA.

(b) Chromatin immunoprecipitation (ChIP) analysis to measure the level of acetylation of histone H3 and H4 in plants with normal (NV) or low levels of DNA methylation (AMT) or plants harvested at the end of a 4-week vernalization treatment (4W + 0), compared to 15-day-old control plants. Histograms marked with the same letter indicate significant differences ($P < 0.01$).

Amasino, 2004), is regulated by DNA methylation. *VIN3* is activated during vernalization but its expression declines rapidly after the end of the cold treatment (Sung and Amasino, 2004). The induction of *VIN3* during vernalization is associated with an increase in the level of acetylation of histones H3 and H4 and of H3 trimethyl-K4 around the promoter and transcription start of *VIN3* (Figure 5b). *VIN3* is not expressed in *AMT* plants with reduced levels of *FLC* expression, indicating that *VIN3* is not regulated by DNA methylation (Figure 5a). Consistent with this, the level of histone acetylation and H3 trimethyl-K4 at the *VIN3* gene in *AMT* plants was comparable to that in non-vernalized plants (Figure 5b).

Our finding that the repression of *FLC* in plants with low methylation is independent of VIN3 activity indicates that the effects of low DNA methylation may act downstream of VIN3 in the vernalization pathway, or may repress *FLC* through a separate pathway.

The downregulation of FLC *in plants with low levels of DNA methylation does not require other genes in the vernalization response pathway*

VRN1 and VRN2 are essential for the complete repression of *FLC* in vernalized plants (Gendall *et al.*, 2001; Levy *et al.*, 2002). VRN2 and VRN1 are required for the methylation of histone H3 at lysines 27 and/or 9, respectively, associated with the downregulation of *FLC* in vernalized plants (Bastow *et al.*, 2004; Sung and Amasino, 2004). To determine whether VRN1 and VRN2 are also required for the demethylation-induced downregulation of *FLC*, we transformed *fca-1 vrn1* and *fca-1 vrn2* mutants with the *AMT* construct (Finnegan *et al.*, 1996) and identified plants with reduced levels of DNA methylation.

FLC expression was substantially reduced in both the *fca-1 vrn1-AMT* and *fca-1 vrn2-AMT* mutant plants with low methylation relative to either non-vernalized or vernalized *fca-1 vrn1* or *fca-1 vrn2* plants with normal levels of DNA methylation (Figure 6a,b). The level of *FLC* expression correlated with the level of DNA methylation; line *fca-1 vrn2-AMT1* has a higher level of both DNA methylation and *FLC* expression than line *fca-1 vrn2-AMT2* (Figure 6b,c). *FLC* expression was repressed in five independent *fca vrn2-AMT* lines and in the only *fca vrn1-AMT* line that had reduced DNA methylation. As *fca1 vrn1* and *fca1 vrn2* are in the ecotype Landsberg *erecta* (L.*er*), we conclude that the effect of demethylation on *FLC* expression in this ecotype is comparable to that seen in C24 lines. The flowering time of these new *AMT* lines was not measured as we have shown previously that reducing DNA methylation in L.*er* results in both repression of *FLC* and activation of *FWA*, another repressor of flowering (Genger *et al.*, 2003).

The observation that the downregulation of *FLC* in plants with low methylation does not require VRN1 and VRN2 activity suggests that the downregulation of *FLC* by reduced DNA methylation occurs through a separate pathway, with both pathways having a similar effect on gene expression and histone modification.

Downregulation of the genes flanking FLC *is associated with decreased histone acetylation and a decrease in H3 trimethyl-K4*

FLC and its two flanking genes are part of a coordinately regulated gene cluster that is repressed in both vernalized plants and in plants with reduced DNA methylation (Finnegan *et al.*, 2004). We found that histone H3 acetylation was decreased upstream of FLC (*UFC*) and *At5g10130* in vernalized plants and in plants with low DNA methylation, just as it was at *FLC* (Figure 4a). The decrease in histone H3 acetylation associated with *UFC* was transient and, 15 days after the end of the cold treatment, the level of histone acetylation associated with this gene was comparable to that in vernalized plants. This transient reduction in histone H3 acetylation of *UFC* correlates with the transient repression of this gene by vernalization (Finnegan *et al.*, 2004). In contrast to *FLC*, there was no change in histone H4 acetylation in the

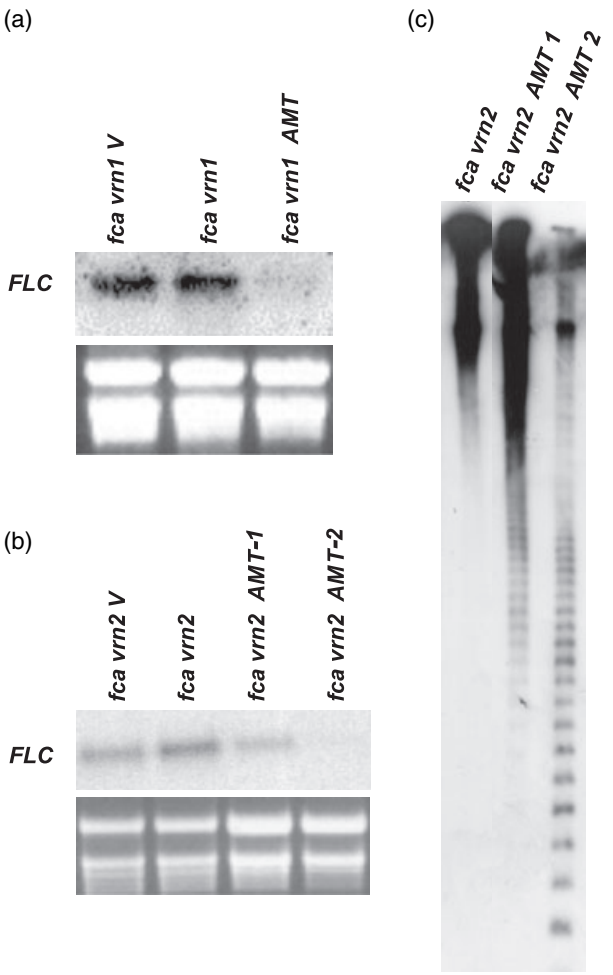

Figure 6. Genes involved in the vernalization pathway are not required for the downregulation of *FLOWERING LOCUS C* (*FLC*) in plants with low levels of DNA methylation.
(a) Northern analysis of RNA isolated from plants vernalized (V) for 4 weeks and harvested 15 days after the cold treatment (V) or control *fca vrn1* plants with normal or low levels of DNA methylation (*AMT*), probed with *FLC*.
(b) Northern analysis of RNA isolated from plants V for 4 weeks and harvested 15 days after the end of the cold treatment (V) or control *fca vrn2* plants with normal or low levels of DNA methylation (*AMT* two independent lines), probed with *FLC*.
(c) Southern analysis of DNA extracted from *fca vrn2* and the two *fca vrn2 AMT* lines, digested with *Hpa*II and probed with the 180-bp centromeric repeat (Martinez-Zapater *et al.*, 1986).

regions of either *UFC* or *At5g10130* examined in our assays (Figure 4a).

The level of H3 trimethyl-K4 around the transcription-translation start of *UFC* and *At5g10130* was decreased both in plants with low levels of DNA methylation and in vernalized plants (Figure 4b). Deacetylation of histone H3 and decreased H3 trimethyl-K4 at both *UFC* and *At5g10130* is a feature common to both *AMT* and vernalized plants.

Reduced genome methylation levels can downregulate an FLC::GUS *transgene but the response depends on chromosomal location*

In eight independent lines, a chimeric transgene including a 6.0-kb region of the *FLC* gene (approximately 2 kb of promoter, the first two exons and the large, first intron) linked to a GUS reporter gene is repressed by vernalization (Sheldon *et al.*, 2002). We crossed these lines with *AMT* plants and found that the response of the transgene varied (Figure 7a–f). In six lines, *FLC::GUS* expression was partially repressed in plants with low methylation, paralleling the behaviour of the endogenous *FLC* gene (Figure 7b,d,g and Figure S2). These lines contained either a single copy of the transgene or several copies at the same insertion site. In the remaining two lines, the behaviour of the *FLC* transgene and the endogenous *FLC* gene differed (Table S2).

In these lines, one with several copies of the transgene arranged in a tandem array (2.9) and the other with a single copy (#7.23), there was little, if any, reduction in *GUS* expression in plants with low methylation compared to the control (Figure 7a,c,h). In contrast to the *FLC::GUS* transgene, the endogenous *FLC* gene was downregulated in the plants with low levels of DNA methylation (Figure 7h) and these plants flowered early. In both lines, the transgene was strongly repressed by vernalization in plants with either normal or low levels of DNA methylation (Figure 7a,e,i; Sheldon *et al.*, 2002). These observations indicate that the response of the transgene to low levels of DNA methylation can be moderated by the sequences flanking the transgene and can differ from its response to vernalization.

Downregulation of FLC *by low levels of DNA methylation does not require sequences within intron 1*

The responses of an *FLC::GUS* transgene to vernalization and to reduced levels of DNA methylation were further differentiated by using the Δ2.8 kb-*FLC::GUS* construct in which a large part of intron 1, spanning bases +419 to +3175, was deleted (Sheldon *et al.*, 2002). The activity of this Δ2.8 kb-*FLC::GUS* transgene is repressed by vernalization but activity recovers during subsequent growth at 21°C. We crossed *AMT* plants with six independent lines carrying the Δ2.8 kb-*FLC::GUS* construct (Figure 3a); in the F2 progeny from four of the six crosses, expression of the Δ2.8 kb-*FLC::GUS* transgene was reduced compared with the progeny of the control cross (Figure 7j). This indicates that the sequences deleted from intron 1 are not required for repression of *FLC* in plants with low DNA methylation. While stable repression of *FLC* by vernalization has an absolute requirement for sequences within intron 1 (Sheldon *et al.*, 2002), we were unable to test whether the low methylation-induced repression of *FLC* is stable as the low methylation phenotype is inherited even in the absence of the *AMT* construct (Finnegan *et al.*, 1996).

The expression of genes in the MADS AFFECTING FLOWERING (MAF*) family of transcription factors is downregulated in plants with low levels of DNA methylation*

The Arabidopsis genome encodes five MADS box genes, *MAF1–5*, that are closely related to *FLC*; the expression of four of these genes is affected by vernalization. *MAF1, MAF3* and *MAF4* are downregulated by vernalization but the extent and kinetics of repression differ from that of *FLC* (Ratcliffe *et al.*, 2001, 2003). Unlike the other *MAF* genes, *MAF5* is induced by vernalization but there is little change in the expression of *MAF2* (Ratcliffe *et al.*, 2003). We found that the expression of *MAF1*, *MAF4* and *MAF5* was strongly downregulated in *AMT* plants, and the expression of *MAF2* and *MAF3* was somewhat lower (Figure 8a). These data suggest that the downregulation of *FLC* and the *MAF* genes in plants with low DNA methylation occurs by a mechanism that is distinct from vernalization and could result from the altered expression of a transcriptional activator common to *FLC* and the *MAF* genes.

The expression of other genes that regulate FLC *transcription is not altered in plants with low methylation*

FLC expression is repressed by genes in the autonomous flowering pathway (Figure 1; Sheldon *et al.*, 1999), raising the possibility that reducing DNA methylation could upregulate a gene(s) in this pathway leading to the downregulation of *FLC*. Mutation of two genes in the autonomous pathway, *FVE* and *FLD*, has been associated with increased acetylation of histones H3 and H4 at *FLC* (Ausin *et al.*, 2004; He *et al.*, 2003; Kim *et al.*, 2004). We monitored the expression of these two genes in *AMT* plants with low *FLC* expression and found no changes in either *FVE* or *FLD* activity (Figure S3). This suggests that the decrease in histone acetylation at *FLC*, observed in AMT plants, is not associated with elevated levels of FLD or FVE. Consistent with this, *FLC* is downregulated in an *fve* mutant with low levels of DNA methylation (Genger *et al.*, 2003). Similarly, the autonomous pathway gene, *FCA*, is not required for the downregulation of *FLC* in plants with low levels of DNA methylation (Figure 6a; Genger *et al.*, 2003).

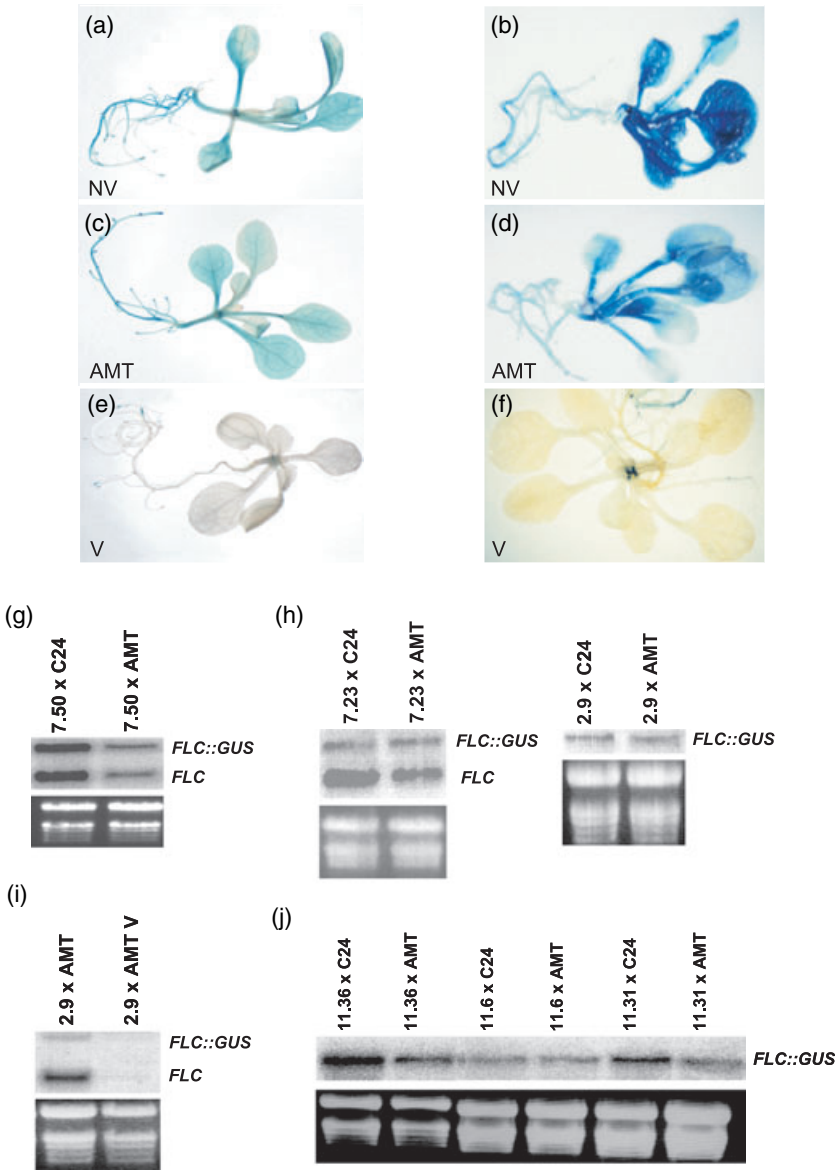

Figure 7. The response of *FLC::GUS* transgenes to low levels of DNA methylation is dependent on their location within the genome.
(a, c and e) Seedlings from plant line 2.9; (b, d and f) seedlings from plant line 7.50. The treatment of the seedlings is indicated on each panel (non-vernalized, NV; vernalized, V; *METI* antisense, AMT).
(g) Northern analysis of RNA isolated from line 7.50 plants with normal (7.50 × C24) or low levels of DNA methylation (7.50 × AMT), probed with *FLOWERING LOCUS C* (*FLC*) and *GUS*.
(h) Northern analysis of RNA isolated from line 7.23 and 2.9 plants with normal (×C24) or low levels of DNA methylation (×AMT), probed with *FLC* and *GUS* (7.23) or *GUS* (2.9).
(i) Northern analysis of RNA isolated from line 2.9 plants with low levels of DNA methylation (2.9 × AMT) with (V) or without a vernalization treatment, probed with *FLC* and *GUS*.
(j) Northern analysis of RNA isolated from three of the six lines tested that carry the Δ2.8 kb *FLC::GUS* construct, 11.36, 11.6 and 11.31, with normal (×C24) or low levels of DNA methylation (×AMT), probed with *GUS*.

Conversely, the downregulation of *FLC* in *AMT* plants could be explained if reducing DNA methylation decreases the expression of a gene(s) required for the activation of *FLC* transcription. A loss of function mutation of any of the following genes is sufficient to prevent or reduce *FLC* expression (Figure 1); genes of the *FRI* gene family, *FRI*, *FRL1* and *FRL2*, *ELF5*, *VIP3*, *VIP4*, *VIP5*, *VIP6/ELF8*, *ELF7*, *ESD4*, *PIE1* and *HUA2* (Doyle *et al.*, 2005; He *et al.*, 2004; Johanson *et al.*, 2000; Michaels *et al.*, 2004; Noh and Amasino, 2003; Noh *et al.*, 2004; Oh *et al.*, 2004; Reeves *et al.*, 2002; Sheldon *et al.*, 1999; Zhang and van Knocker, 2002; Zhang *et al.*, 2003). A subset of these genes is known to affect *MAF* gene expression in addition to their effect on *FLC* (Doyle *et al.*, 2005; He *et al.*, 2004; Oh *et al.*, 2004). There was no change in the expression of any of these positive regulators of *FLC* in plants with low CpG methylation (Figure 8b and Figure S3). Therefore, the downregulation of *FLC* in plants with low levels of DNA methylation is not due to the reduced expression of any of the known enhancers of *FLC* transcription.

Discussion

The activation and repression of *FLC* expression is modulated by proteins known to play a role in modifying chromatin structure (Gendall *et al.*, 2001; He *et al.*, 2004; Noh and Amasino, 2003; Oh *et al.*, 2004), making the *FLC* locus an excellent system for studying epigenetic regulation of gene expression. *FLC* is part of a small coordinately regulated gene cluster suggesting that changes in chromatin structure

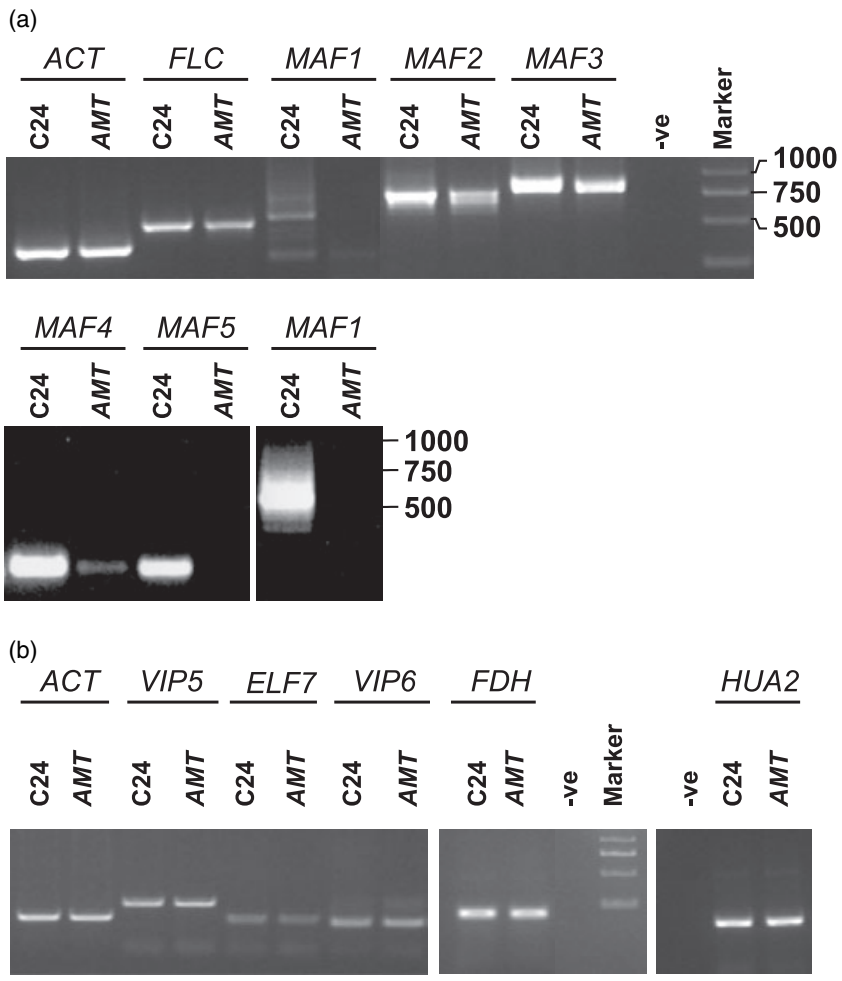

Figure 8. Expression of the *MADS AFFECTING FLOWERING* (*MAF*) gene family is repressed in *AMT* plants but the expression of transcriptional enhancers, common to *FLOWERING LOCUS C* (*FLC*) and the *MAF* genes, is not affected.
(a) Reverse-transcription polymerase chain reaction (RT-PCR) analyses showing that the expression of *FLC* and *MAF1–5* is reduced in plants with low levels of DNA methylation (*AMT*) compared with plants with normal levels of DNA methylation (C24). The expression of *MAF1–5* was also repressed in *AMT* plants in the Landsberg *erecta* (L.*er*) background (not shown).
(b) RT-PCR analyses showing that the expression of *VERNALIZATION INDEPENDENT 5* (*VIP5*), *VIP6/ EARLY FLOWERING 8* (*ELF8*), *ELF7* and *HUA2* is not altered in plants with low levels of DNA methylation (*AMT*) compared to plants with normal levels of DNA methylation (C24).

may also affect the expression of its flanking genes (Finnegan *et al.*, 2004). We have previously shown that *FLC* expression is repressed in plants with decreased levels of another epigenetic modifier, namely DNA methylation (Sheldon *et al.*, 1999). Here, we show that CpG methylation plays a role in regulating *FLC* expression, but that CpNpG and asymmetric methylation are not involved. This regulation does not involve demethylation of the *FLC* gene itself, as the only methyl-CpG (^{m}CpG), cytosine$_{2360}$, can be deleted (in the Δ2.8 kb-*FLC::GUS* construct) without affecting the repression of *FLC* in plants with reduced DNA methylation. Furthermore, the repression of *FLC* does not result from hypermethylation of the regulatory sequences of this gene in plants with reduced levels of DNA methylation (Figure 3b). Taken together, these data suggest that DNA methylation may be regulating the expression of a regulator of *FLC*, rather than *FLC* itself. Consistent with this, we found that the five *FLC*-related *MAF* genes are also downregulated in plants with low levels of DNA methylation (Figure 8a).

FLC is also repressed by vernalization (Michaels and Amasino, 1999; Sheldon *et al.*, 1999), but the reduced activity is not associated with any change in DNA methylation in the *FLC* gene (Table S1). The repression of *FLC* by vernalization is associated with methylation of histone H3 at lysines 9 and 27 (Bastow *et al.*, 2004; Sung and Amasino, 2004). When both these modifications occur on the same histone, CMT3 methyltransferase can bind, resulting in methylation of the adjacent DNA (Lindroth *et al.*, 2004). However, we found no role for CMT3 in the vernalization response suggesting either that these modifications do not occur on the same histone molecule in vernalized plants or that these modifications are not sufficient for the recruitment of CMT3.

In both vernalized plants and plants with low levels of DNA methylation, the expression of *UFC* and *At5g10130*, the two genes that flank *FLC*, is also repressed. We have shown that the downregulation of all three genes is associated with a decrease in histone H3 acetylation across a region of about 750–800 bases of each gene, extending from the promoter to 3′ of the translation start. This change in histone acetylation occurs in both vernalized plants and plants with low levels of DNA methylation (Figure 4a), but we do not know whether deacetylation is a cause or a consequence of repression of these genes. The reduction in acetylation of histone H3 at

UFC following vernalization was transient as was the repression of *UFC* expression by vernalization (Finnegan *et al.*, 2004). Both vernalization and reduced DNA methylation are associated with decreased histone H4 acetylation in the region of *FLC* spanning the start of transcription and translation, but not in the upstream region. We saw no change in histone H4 acetylation, under either condition, within the regions of *UFC* or at A*t5g10130* monitored in our assay. We do not know what causes histone deacetylation in the *FLC* cluster but it is likely that this is an active process involving a histone deacetylase(s). The same deacetylase enzyme(s) may be involved in reducing acetylation at these genes in both vernalized plants and plants with low DNA methylation.

The level of histone H3 trimethyl-K4 across the translation start of each gene was also decreased in both vernalized and *AMT* plants (Figure 4b). Loss of H3 trimethyl-K4 is probably a consequence of the reduced transcriptional activity of these genes as hypermethylation of K4 within the mRNA coding region is indicative of recent transcriptional activity (Ng *et al.*, 2003). The reduction in the level of H3 trimethyl-K4 is likely to occur by histone replacement, perhaps during DNA replication, as there is no known mechanism causing demethylation of lysine residues that carry three methyl groups (Shi *et al.*, 2004).

While the response of *FLC* to vernalization appears to be largely independent of its genomic location, our data suggest that the repression of *FLC* by low levels of DNA methylation can be markedly influenced by flanking chromatin. The repression of *FLC* in vernalized plants is associated with another histone modification, methylation of histone H3 at lysines 9 and 27 (Bastow *et al.*, 2004; Sung and Amasino, 2004); methylation of histone H3 at these residues may facilitate repression of *FLC* in any genomic location. VRN1 and VRN2 are not required for the downregulation of *FLC* by reduced DNA methylation suggesting that there is no methylation of lysines 9 and 27 of H3 under these conditions, perhaps making *FLC* activity more susceptible to the influence of flanking chromatin.

Despite the similarity in histone modification of the *FLC* gene cluster seen in vernalized plants and plants with low levels of DNA methylation, other observations suggest that the regional repression of gene expression, in response to these agents, is achieved by different mechanisms. The known components of the vernalization response pathway (VIN3, VRN1,VRN2 and sequences within the *FLC* intron) are not required for the downregulation of *FLC* by reduced DNA methylation. Our data support the idea that the downregulation of *FLC* in plants with low levels of DNA methylation is associated with altered expression of a transcriptional regulator (either an activator or a repressor) that is common to *FLC* and the five members of the *MAF* family. The Arabidopsis Paf1 complex is required for expression of *FLC* and the *MAF* genes (He *et al.*, 2004; Oh *et al.*, 2004), but we found no change in either the expression of four known members of this complex, *VIP4–6* and *ELF7*, or of *HUA2* (Figure 8b), another transcriptional regulator that affects expression of the *FLC* and *MAF* genes (Doyle *et al.*, 2005). The possibility remains that DNA methylation modulates the expression of a regulator of both *FLC* and the *MAF* genes, which may or may not act through the Paf1 complex.

In summary, we have demonstrated that the downregulation of *FLC* in plants with low levels of DNA methylation occurs by a pathway that differs from the vernalization pathway. *FLC* is not regulated directly by DNA methylation in either vernalized plants or in plants with low levels of DNA methylation. Of the two epigenetic control pathways, vernalization plays a role in regulating flowering in response to environmental cues, but the biological significance of the pathway involving the control of *FLC* and the *MAF* genes by DNA methylation is unknown. We propose that DNA methylation modulates the expression of a transcriptional regulator common to both *FLC* and the *FLC*-like *MAF* gene family, resulting in low *FLC* expression and maintenance of the chromatin of the *FLC* region in an inactive state.

Experimental procedures

Plant lines

The *AMT* plants used in these experiments are derivatives of the *METI* antisense line C24 10.5 described in Finnegan *et al.* (1996); this line is homozygous for three copies of the antisense transgene, located at a single insertion site. Other *AMT* lines were generated for this work using the same *METI* antisense construct. The *CMT3* RNAi construct was introduced into the L.*er fca-1* mutant; this genotype was chosen because it has elevated *FLC* expression and another member of the CMT family, *CMT1*, is inactivated (Henikoff and Comai, 1998). Two independent RNAi lines with a substantial decrease in DNA methylation were identified; neither line showed a change in the level of *FLC* expression compared to the appropriate NV or V control. The analysis of flowering time and *FLC* expression was done in plants from the T3 generation, selected on kanamycin. The *ddm1-1* mutant was from the seventh generation of self-progeny and had a late flowering phenotype.

Plant transformation

Plant transformation was carried out by the floral dip method (Clough and Bent, 1998). Transgenic T1 plants were identified by plating on Muroshige and Skoog (MS) medium containing kanamycin sulphate (30 μg ml^{-1}). Plants with reduced levels of DNA methylation were identified by Southern analyses in which DNA, isolated from leaves (Taylor *et al.*, 1989), was cleaved with *Hpa*II (for plants carrying the *AMT* construct) or *Msp*I (for plants transformed with the *CMT3* RNAi construct) and probed with the centromeric repeat sequence (Martinez-Zapater *et al.*, 1986), as described in Finnegan *et al.* (1996). *Hpa*II cleaves the sequence CCGG when the internal cytosine is unmethylated and the external cytosine is either unmethylated or hemimethylated (Nelson and McClelland, 1991). *Msp*I cleaves the same sequence but is inhibited by methylation of the external cytosine (Nelson and McClelland, 1991).

DNA and RNA isolation

DNA was isolated from leaf material using the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction procedure described in Taylor *et al.* (1989). Total RNA was extracted from approximately 0.5 g of tissue as described (Dolferus *et al.*, 1994).

Southern and Northern hybridizations

Southern hybridizations were done as described in Finnegan *et al.* (1996). RNA gel blots assays were done using 10 μg total RNA as described in Sheldon *et al.* (1999).

Bisulphite sequencing

DNA for bisulphite sequencing was treated according to Kishimoto *et al.* (2001). Polymerase chain reaction (PCR) fragments amplified from bisulphite DNA were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and cloned into a plasmid vector before sequencing. Sequencing was done using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Branchburg, NJ, USA). Sequence reactions were run on an ABI Prism DNA Sequencer Model 377 and analysed using ABI PRISM DNA SEQUENCING SOFTWARE VERSION 2.1.2. The GCG sequence analysis package version 8.1 was used for nucleotide comparisons. The sequences of the primers used are presented in Table S3.

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were done according to Johnson *et al.* (2002). Antibodies recognizing acetylated histone H3 (K9, K14), tetra-acetylated H4 (K5, K8, K12, K16) and H3 trimethyl-K4 were purchased from Upstate Biotechnology (Charlottesville, VI, USA). The data presented represent the average of at least three replicate experiments.

Real-time PCR

The amount of DNA precipitated in ChIP assays was quantified by real-time PCR using a Rotor-Gene 2000 Real-Time Cycler (Corbett Research, Sydney, Australia). Cycling and reaction conditions were as described in Klok *et al.* (2002). In each experiment, the amount of DNA precipitated from the test sample (V or *AMT* plants) was compared to the DNA precipitated by the same antibody from a control, 15-day-old NV C24 plants. The relative of amounts of DNA precipitated between the test and control samples was determined using the 'Comparative Quantification' analysis method (ROTOGENE-5 software, Corbett Research). The method uses information about the start of the exponential phase of amplification (Take-off Point) and the average reaction efficiency of the samples to be compared, to enable direct concentration comparisons between the different samples, generating a relative concentration. The housekeeping gene *S-ADENOSYL METHIONINE SYNTHASE* (*SAM*; At4g01850) whose expression does not change following vernalization (Finnegan *et al.*, 2004) or in plants with low levels of DNA methylation was used to normalize the amount of DNA precipitated in each sample, and the ratio between test and control for the gene of interest was determined. A ratio of 1 indicates no change from the control whereas ratios of <1 indicate a reduction in histone acetylation and ratios of >1 indicate an increase in histone acetylation relative to the control. Quantification for each primer set and immunoprecipitated DNA template combination were performed in triplicate and included a no template control to ensure that the results were not influenced by primer-dimer formation. The primers used for real-time PCR analysis are given in Table S4.

To verify our data, we also used duplex PCR followed by Southern hybridization to determine the level of histone acetylation in some samples. For this analysis, the *ACTIN2/7* gene was used for normalization. The data obtained using this method were comparable to those obtained with real-time PCR. For plants V for 4W then harvested 15 days after the end of the cold treatment (4W +15), the level of H3 histone acetylation was 0.56 (V/NV) and histone H4 acetylation was 0.59 (V/NV); for *AMT* plants the corresponding values were 0.4 (H3 acetylation *AMT*/C24) and 0.5 (H4 acetylation *AMT*/C24). These values are the average of three replicate experiments.

Statistical analysis

The data for both ChIP and reverse-transcription (RT) PCR assays are presented as the average of a ratio of ratios. Because these data may not be distributed normally, it is not appropriate to present either the standard deviation or standard error about the mean (Pfaffl, 2002). Significance was tested using the non-parametric Mann–Whitney *U*-test, which does not rely on the data being normally distributed.

Staining for GUS activity

The protocol used for staining for GUS activity can be found in Sheldon *et al.* (2002).

Reverse-transcription polymerase chain reaction

RT-PCR was done using an Access RT-PCR system (Promega, Madison, WI, USA) according to the manufacturer's specifications. All reactions were done with an annealing time of 60°C and the number of cycles was adjusted so that the reactions remained within the linear range. The primer pairs and number of cycles for the different genes are given in Table S5.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Upstream regions of the *FLC* gene have different levels of histone H4 acetylation following vernalization.

Figure S2. The expression of the 6.0-kb *FLC::GUS* transgene at some genomic locations is reduced in plants with low levels of DNA methylation.

Figure S3. The expression of genes that regulate *FLC* expression is not altered in plants with low levels of DNA methylation.
Table S1 There is no change in DNA methylation in the regulatory regions of *FLC* in vernalized plants
Table S2 The *FLC::GUS* transgene was inserted at different genomic locations
Table S3 The sequence of primers used for the bisulphite sequencing of FLC
Table S4 The sequence of primers used for Real-Time PCR analysis of DNA precipitated in chromatin immunprecipitation assays.
Table S5 The sequence of primers used to examine the expression of genes that regulate *FLC*
This material is available as part of the online article from http://www.blackwell-synergy.com

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