Molecular Cell, Vol. 6, 791–802, October, 2000, Copyright ©2000 by Cell Press

The Late Flowering Phenotype of *fwa* Mutants Is Caused by Gain-of-Function Epigenetic Alleles of a Homeodomain Gene

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Summary

The transition to flowering in *Arabidopsis thaliana* is delayed in *fwa* mutant plants. *FWA* was identified by loss-of-function mutations in normally flowering revertants of the *fwa* mutant, and it encodes a homeodomain-containing transcription factor. The DNA sequence of wild-type and *fwa* mutant alleles was identical in the genomic region of *FWA*. Furthermore, the *FWA* gene is ectopically expressed in *fwa* mutants and silenced in mature wild-type plants. This silencing is associated with extensive methylation of two direct repeats in the 5′ region of the gene. The late flowering phenotype, ectopic *FWA* expression, and hypomethylation of the repeats were also induced in the *ddm1* hypomethylated background. Mechanisms for establishment and maintenance of the epigenetic mark on *FWA* are discussed.

Introduction

Induction of flowering at the appropriate moment is essential for many plant species to reproduce successfully. The fine tuning of the transition from the vegetative to the reproductive phase is believed to be under control of multiple factors. These are both endogenous, such as gibberellins and carbohydrate metabolites, and environmental, such as day length, temperature, and light quality. To understand this process, a genetic approach is underway in *Arabidopsis* in which a multitude of mutants influencing the timing of flowering are being studied. The combination of physiological, genetic, and molecular approaches using these mutants has led to a model of floral induction that consists of a photoperiod promotion pathway, a vernalization promotion pathway, and an autonomous promotion pathway (Koornneef et al., 1998b; Levy and Dean, 1998; Simpson et al., 1999). The cloning and molecular characterization of several of the involved genes are allowing a molecular interpretation of these pathways. However, the available information is fragmented, and many aspects of this developmental process remain poorly understood.

One of the factors suggested to play a role in the regulation of gene expression affecting flowering transition is DNA methylation (Finnegan et al., 2000). The actual significance of DNA methylation for gene regulation in plant development remains unknown. An overall reduction in total genomic cytosine methylation of up to 70% has been found in transgenic plants with reduced amounts of DNA methyltransferase (Finnegan et al., 1996; Ronemus et al., 1996) and in *d*ecrease in *D*NA *m*ethylation (*ddm1*) mutant plants that are defective in a protein that is likely to be involved in chromatin remodeling (Jeddeloh et al., 1999). Such plants develop a number of phenotypic abnormalities (Vongs et al., 1993; Finnegan et al., 1996; Kakutani et al., 1996; Ronemus et al., 1996). Furthermore, it has been observed that stable enhancement of the methylation level in specific genes can suppress expression of these genes, leading to mutant phenotypes (Jacobsen and Meyerowitz, 1997; Cubas et al., 1999; Jacobsen et al., 2000).

In relation to flowering, experimental arguments supporting a role for DNA methylation are largely correlative (Finnegan et al., 1998). For instance, *Arabidopsis* plants that are exposed to low temperatures during a prolonged period (vernalization), and plants that are treated with the DNA demethylating agent 5-azacytidine show reduced levels of 5-methylcytosine and early flowering as compared to untreated plants (Burn et al., 1993). Thus, it has been hypothesized that vernalization promotes flowering through demethylation of the genome. Apart from early flowering plants, late flowering plants were derived from the hypomethylated backgrounds of antisense DNA methyltransferase (as-*MET1*) (Ronemus et al., 1996) and *ddm1* (Kakutani et al., 1996). Therefore, contrasting phenotypes have been related to altering methylation, suggesting that multiple genes with opposite effects might be involved in the epigenetic regulation of flowering. Nevertheless, to prove and understand the involvement of such mechanisms awaits the identification of target genes that are affected directly by methylation.

The late flowering trait induced by *ddm1* hypomethylation background was genetically mapped to the chromosomal region containing *FWA* (Kakutani, 1997), a well-characterized flowering time gene. The *fwa* mutant is delayed in the transition to flowering and is semidominant, unlike most flowering time mutants (Koornneef et al., 1991). Based on double mutant genetic and physiological analyses, *FWA* is presumed to affect flowering through the speculated photoperiod promotion pathway in the current model for the control of flowering initiation (Koornneef et al., 1998b). In addition to its function in

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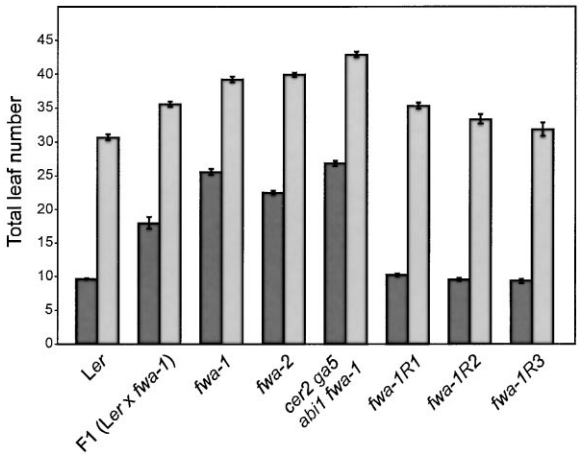


Figure 1. Flowering Time of the Different *FWA* Alleles

Mean flowering time (measured as the total number of leaves produced by the plant before flowering) of 10–15 plants grown under LD conditions (black bars) or under SD conditions (gray bars) is shown. The standard error of the mean is indicated on each bar.

the transition from the vegetative to the reproductive meristem, several observations indicate that *FWA*, together with the recently cloned flowering promoting gene *FT*, also plays a role in the control of flower meristem identity (Ruiz-Garcia et al., 1997; Nilsson et al., 1998; Roldán et al., 1999; Onouchi et al., 2000). It has been suggested that *FWA* and *FT* affect meristem identity in a pathway that operates parallel to that of the well-characterized *LFY* gene (Kardailsky et al., 1999; Kobayashi et al., 1999).

In this work, we describe the molecular identification of the *FWA* gene and show that the late flowering phenotype of *fwa* mutants is caused by gain-of-function epi alleles that lack methylation in two repeated sequences located in the 5′ region of *FWA*.

Results

Characterization of *FWA* Mutants

Two different *fwa* mutant alleles have previously been described (Koornneef et al., 1991); *fwa-1* was induced by ethyl methanesulphonate (EMS) and *fwa-2* by fast neutrons. Plants carrying these *fwa* mutations flower later than Landsberg *erecta* (L*er*) wild-type plants (Figure 1). This delay in flowering is relatively stronger under long day (LD) than under short day (SD) light conditions. Plants heterozygous for the *fwa* mutation flower intermediate between wild-type and the homozygous *fwa* mutant plants, indicating that *fwa* alleles are semidominant (Figure 1).

To determine whether the dominance of *fwa* mutations is due to gain-of-function of these alleles, we attempted to obtain intragenic suppressor mutations of *fwa* that show a wild-type-like phenotype. Seeds of the *fwa-1* marker line carrying the mutations *cer2 ga5 fwa-1 abi1* were γ irradiated, and approximately 5000 M2 plants were screened under LD conditions for altered flowering time. Five early flowering plants were obtained and crossed with the L*er* wild-type to try to separate the new mutation causing the early flowering from the mutations of the marker line. Three of these revertant plants (named *fwa-1R1*, *fwa-1R2,* and *fwa-1R3*) gave rise to F1 hybrids that flowered early. In addition, no late flowering plants were observed in F2 progenies of 356 plants, and therefore these revertants are likely to carry intragenic suppressor mutations in the *fwa* mutant gene. Figure 1 shows the flowering time, under LD and SD conditions, of the revertants and the marker line from which they were derived. These results strongly suggest that *fwa* mutants carry gain-of-function alleles of the *FWA* gene, while the second site mutations *fwa-1R1*, *fwa-1R2*, and *fwa-1R3* result in loss-of-function alleles of *FWA*.

Positional Cloning of *FWA*

FWA is located on chromosome 4 between the two morphological markers *ga5* and *emb35*, which are 6.2 cM apart. From a mapping population of 1306 plants, 120 recombinants were identified between these two markers. Two of them had crossovers between *GA5* and *FWA*, indicating that *FWA* maps only 0.1 cM from *GA5*, while 118 had crossovers between *FWA* and *EMB35*. The location of *FWA* was further refined with molecular markers located within this region (Figure 2A).

Several YACs were selected from the published YAC contig of chromosome 4 (Schmidt et al., 1995), and their relative positions in relation to the molecular markers were further refined. Thus, *FWA* could be located in a region of about 60 kb between markers CC128 and pcr28. A genomic library was made from *fwa-1* in a binary cosmid vector that was screened with the YAC clone EG1F12 containing both markers CC128 and pcr28. The positive cosmid clones from this screen were arranged into a contig (Figure 2B) and used as markers in RFLP analysis, which indicated that clones WS20 and WS94 did not have any crossovers left with the *FWA* locus.

Nine overlapping cosmids (see Figure 2B), spanning the region between CC128 and pcr28, were used to transform wild-type plants, and between 16 and 48 transformed plants were generated per cosmid. Late flowering was only observed in plants transformed with the two overlapping cosmids WS20 and WS28. Respectively, 5 out of 47 and 14 out of 48 plants transformed with WS20 and WS28 flowered significantly later than L*er*, indicating that *FWA* is on the overlap of these two cosmids. The DNA sequence of this region for the Columbia (Col) accession was obtained from the *Arabidopsis thaliana* database and showed that the overlap of these two cosmids contains only one complete predicted gene, which encodes a homeodomain (HD) transcription factor (Figures 2C and 3).

A 5.3 kb region corresponding to this HD gene was sequenced in the L*er* wild-type, the two *fwa*, and three revertant alleles to look for mutations. The three revertant alleles all contained different mutations within the open reading frame of this gene; *fwa-1R1* and *fwa-1R3* both have a single base pair deletion causing a premature stop of translation, and *fwa-1R2* has a single base pair change resulting in a glycine to arginine transition (Figure 3A). However, the sequences of wild-type and both mutant alleles were identical, indicating that

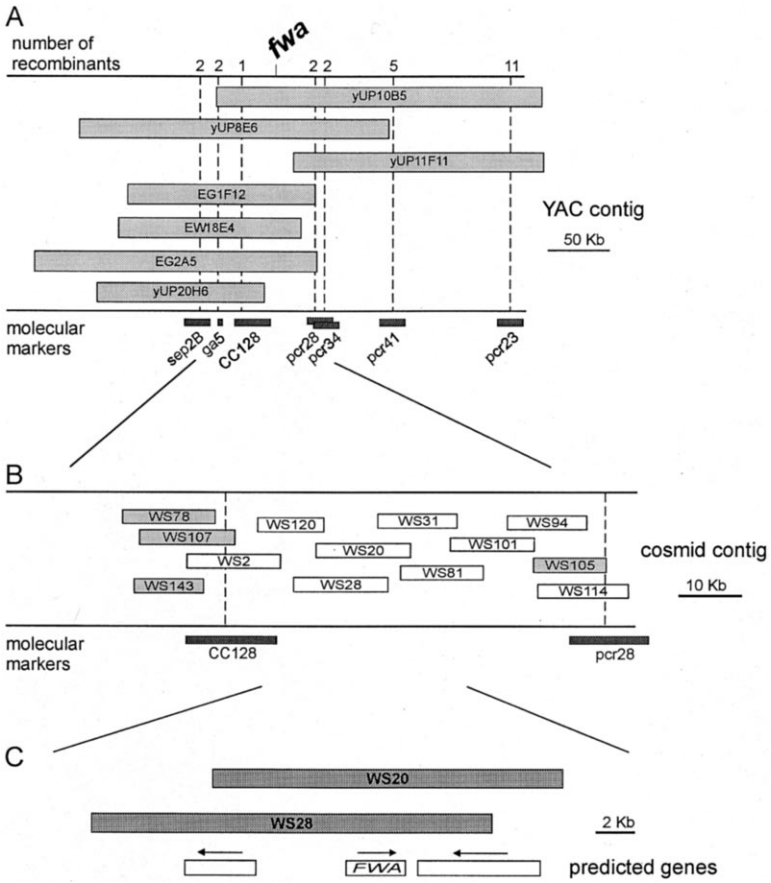


Figure 2. Molecular Markers, YACs, and Cosmids in the *FWA* Region

(A) The YAC contig and molecular markers used to locate the *FWA* locus. The number of recombinants between every molecular marker and *FWA* is indicated in the top.

(B) The cosmid contig; white-colored cosmids were used for plant transformation experiments.

(C) Predicted genes in the overlap of cosmids WS20 and WS28. Arrows above the genes show the direction of transcription.

the cause of the *fwa* mutant phenotype cannot be due to mutations in the *FWA* gene itself.

Analysis of expression of this HD gene in *fwa* mutants showed that this is altered in both *fwa* mutant alleles as compared to L*er* wild-type plants. However, expression of other genes in the region did not show differences between *fwa* and wild-type plants (data not shown). Therefore, we conclude that the late flowering of *fwa* mutants is due to a direct regulation of this HD gene, which is considered to be the *FWA* gene.

To explain the upregulation of *FWA* in *fwa* mutants we further analyzed the structure and expression of this gene. The complete cDNA of *FWA* was obtained by RACE-PCR from total RNA of the *fwa-1* mutant because we could not detect any cDNA for this gene in wild-type cDNA libraries, nor in EST databases. Comparison of the cDNA with the genomic sequence showed that *FWA* contains 10 exons (Figure 3A). The predicted translation start is in the third exon, the first two exons being located 700 base pairs upstream of this start. The cDNA encodes a predicted protein of 686 amino acids. A database search with this putative *FWA* protein sequence revealed strong homology with proteins belonging to the subclass of plant HD-ZIP homeodomain proteins, named HD-GL2 (homeodomain Glabra2) (Rerie et al., 1994; Lu et al., 1996). The highest homology of *FWA* was found with ANTHOCYANINLESS2 (ANL2) (Kubo et al., 1999). *FWA* showed all the characteristics of HD-GL2 transcription factors; the presence of a homeodomain in the N-terminal part followed by a leucine zipper (Di Cristina et al., 1996), and a StAR-related lipid-transfer (START) domain (Ponting and Aravind, 1999). As shown in Figure 3B, comparison of the putative *FWA* protein with ANL2 and two other members of the HD-GL2 class revealed amino acid conservation throughout the whole protein. Homology was especially strong in the regions of the homeodomain and the START domain but weaker at the amino terminus.

An interesting feature of the *FWA* genomic sequence was the presence of two direct repeats in the 5′ region, one of 38 base pairs with 100% homology, and one of 198 base pairs with 94% homology (Figure 3A). The small repeat was located in the promoter region of *FWA*, while the larger one covered the first two exons and part of the first two introns. Consequently, the cDNA contains a direct repeat of 56 base pairs (with 91% homology) in the 5′ untranslated leader. The two repeats appear to be unique in the *Arabidopsis* genome because homologous sequences could not be found in the databases.

Expression of the *FWA* Gene

The expression of *FWA* was analyzed in different *FWA* genetic backgrounds by Northern blot hybridization. RNA was extracted from whole plants of various ages, which were grown under LD and SD light conditions. The two *fwa* mutant alleles showed a similar expression

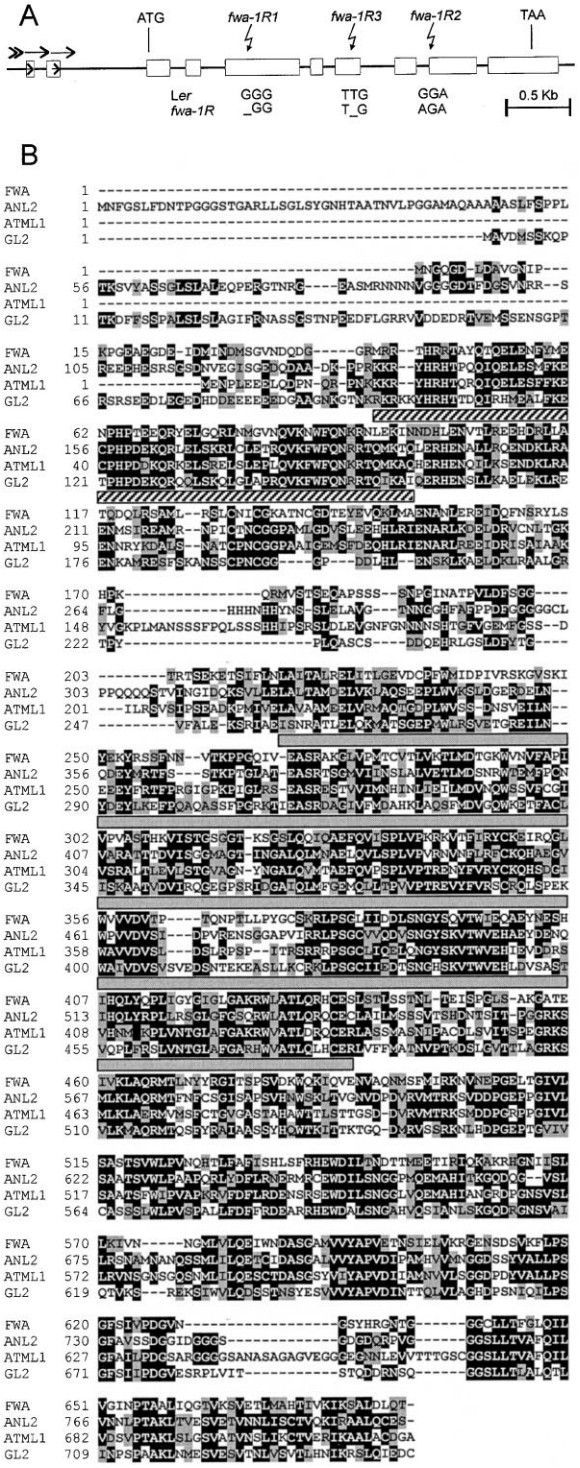


Figure 3. Structure of the *FWA* Gene and the Protein that It Encodes

(A) Schematic representation of the *FWA* gene. Open boxes represent exons. The start codon (ATG), stop codon (TAA), and the position and nature of the mutations in the three revertants are indicated. The arrows above the 5' region mark the two direct repeated sequences, while arrows within the first two exons show the position of the direct repeat in the untranslated leader of the mRNA.

(B) The deduced amino acid sequence of the *FWA* protein compared with ANL2 (GenBank accession number AF077335), ATML1 (U37589), and GL2 (L32873). Identical amino acids are shaded in black, and conservative changes are shaded in gray. The homeodomain is underlined with a hatched box and the START domain with a gray box.

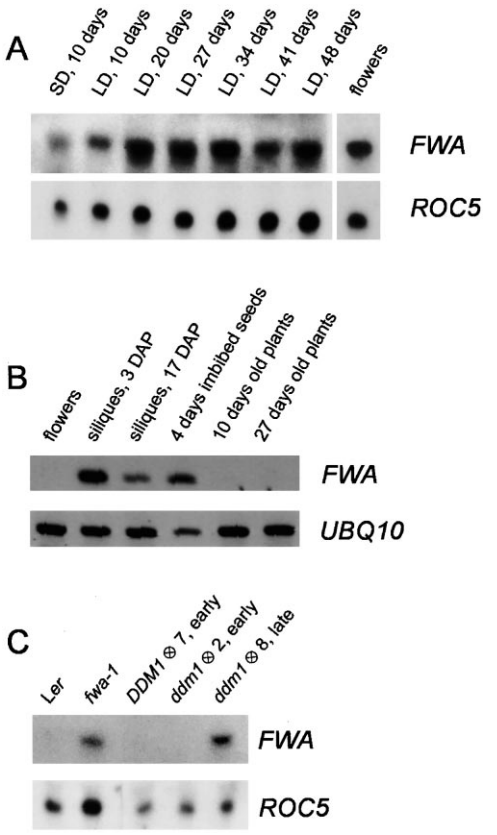


Figure 4. Expression of *FWA*

(A) Northern blot analysis of *FWA* expression in *fwa-1* plants. Plants were grown under SD or LD conditions, and total RNA was extracted from flowers and complete plants 10, 20, 27, 34, 41, and 48 days after planting. The blot was probed with a fragment of the *ROC5* gene as a loading control.

(B) Analysis of *FWA* expression in different tissues or complete plants of wild-type L*er* by RT–PCR with *FWA* gene-specific primers. A fragment of the *UBIQUITIN10* gene was amplified as a control.

(C) Northern blot analysis of *FWA* expression in different early and late flowering *DDM1* lines that were self-fertilized for two, seven, or eight generations. Plants were grown under LD conditions, and total RNA was extracted 3 weeks after planting. The blot was probed with a fragment of the *ROC5* gene as a loading control.

pattern of *FWA*. Transcripts were present through the full life cycle of the plant and in different plant organs, including flowers (Figure 4A). In contrast, no expression could be detected in wild-type and revertant alleles.

To detect whether the transcript might be present at a very low level in wild-type plants, RT–PCR was used. The transcript could not be detected in RNA isolated from whole plants at vegetative or reproductive phases. However, it could be detected in siliques of different ages, from 3 days after pollination (DAP) until maturity of the seeds and in germinating seeds (Figure 4B).

FWA expression was analyzed by Northern blot hybridization in 3-week-old plants of the late flowering

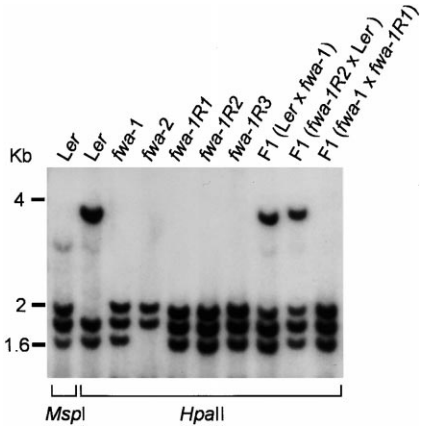


Figure 5. Southern Analysis of Methylation Patterns in Different *FWA* Allele Backgrounds

Genomic DNA of plants with different *FWA* alleles and of F1 plants from crosses between different alleles was digested with MspI or HpaII and hybridized with cosmid WS31 as a probe. In wild-type, a DNA fragment of about 4 kb, is cut by MspI but not by HpaII. However, this fragment is cut by HpaII in *fwa* mutant and *fwa-1R* revertant alleles, indicating a loss of methylation.

mutants *fca-1*, *fve-1*, *co-3*, *gi-1*, and *ft-1* and of the floral meristem identity mutant *lfy-6*. The *FWA* transcript could not be detected in any of these mutants (data not shown). Furthermore, *FWA* RNA could not be detected in 3-week-old plants of different early flowering (Col and Wassilewskija), middle late flowering (Fukuyama and Llagostera) and late flowering (Canary Islands and Saint Feliu) accessions (data not shown).

DNA Hypomethylation in the *fwa* Mutants

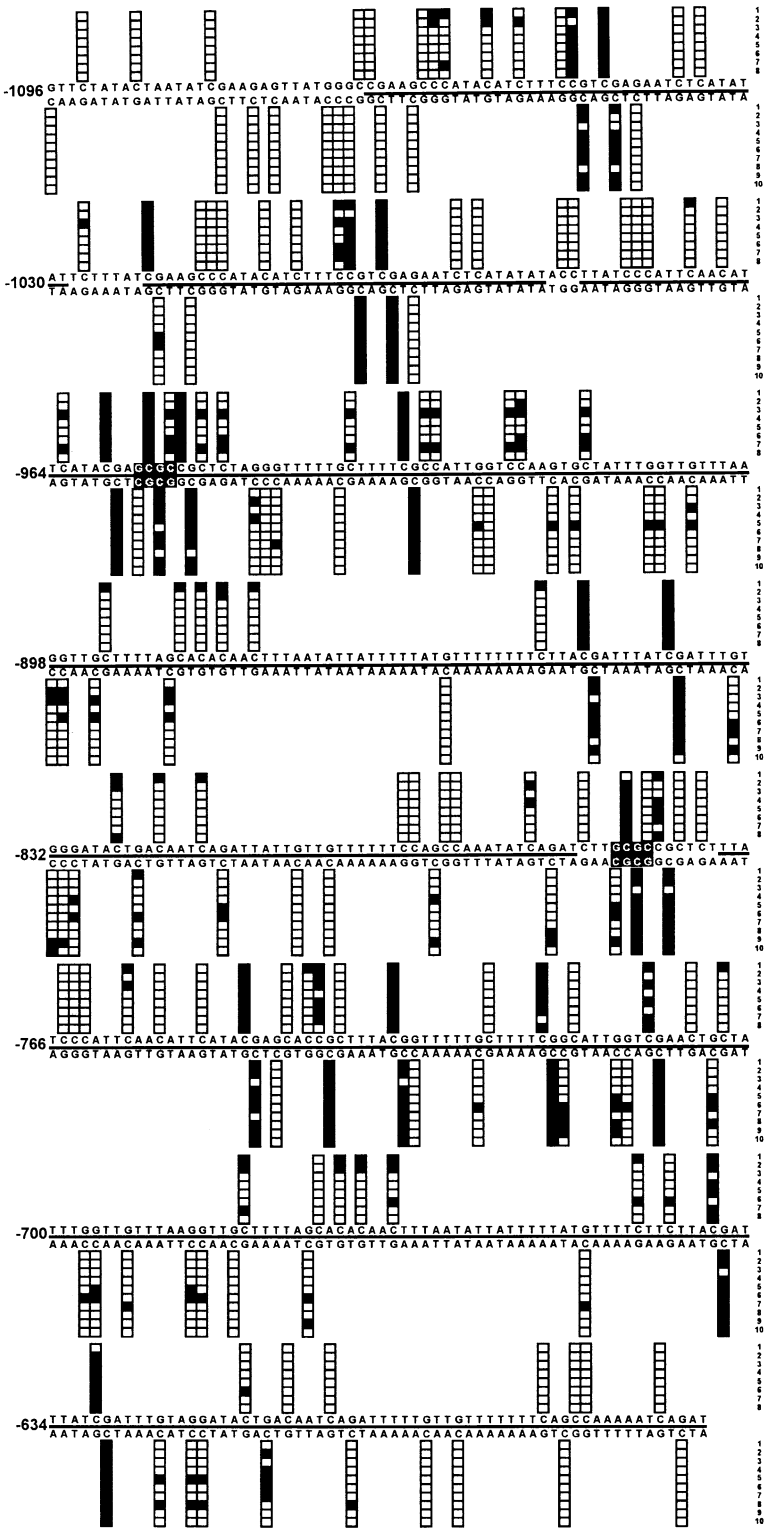
During the map-based cloning of *FWA*, several RFLPs between wild-type and *fwa* mutant DNA were detected with methylation-sensitive restriction enzymes. Southern hybridization of genomic DNA cleaved with the isoschizomeric enzymes HpaII and MspI was used to examine whether there was a difference in DNA methylation level between *fwa* mutants and wild type. Hypomethylation in the *fwa* mutant was found with 14 probes that were located in a region of 5 Mb, surrounding the *FWA* locus (an example of this hypomethylation is shown in Figure 5). Five single copy probes located elsewhere in the genome did not show a difference in DNA methylation levels. Furthermore, *fwa-1* and *fwa-2* do not have an identical methylation pattern, and the three revertants show the same methylation pattern as *fwa-1*, from which they derived. In addition, plants that are heterozygous for *fwa* show both the methylated and the unmethylated restriction sites, suggesting that the wild-type *FWA* allele is normally methylated and the mutant *fwa* allele is hypomethylated (Figure 5). The genomic DNA methylation status was also analyzed in repeated sequence regions outside the *FWA* locus using probes for the 180 bp centromere repeats (Martínez-Zapater et al., 1986), rDNA (Ronemus et al., 1996), and the retrotransposon Ta3 (Konieczny et al., 1991; Kakutani et al., 1999). In all cases, the same methylation pattern was observed in *fwa* and wild-type DNA (data not shown). Therefore, the hypomethylation of *fwa* seems to be restricted to the region of the *FWA* locus. These observations prompted us to further investigate methylation as a possible cause for the upregulation of *FWA* expression in *fwa* mutants.

Inverted repeats and multiple-copy sequences have been shown to be more sensitive to methylation and gene silencing than single-copy sequences (Jacobsen, 1999). Therefore, we looked in detail at the cytosine methylation status of the repeated sequences located in the 5′ region of the *FWA* gene (Figure 3A). Using bisulfite sequencing (Jacobsen et al., 2000) on DNA isolated from whole rosettes at the vegetative phase, we analyzed a region of approximately 1.4 Kb containing the two direct repeats just upstream of the translation start site. In wild-type plants, methylation was restricted to the repeats only and found at all 20 CG sites. Analysis of 8 top-strand and 10 bottom-strand clones revealed that within the wild-type repeats, 89% of cytosines in symmetric CG sequences context are methylated (Figure 6). However, methylation is not restricted to these symmetric sites, cytosines in a nonsymmetric context were also methylated 13% of the time (Figure 6). Furthermore, we found wide variation in cytosine methylation between individual clones. The pattern of non-CG methylation seems to be variable with little preference for sequence context. Analysis of five top- and three bottom-strand clones of *fwa-1* showed complete bisulfite conversion, indicating that no cytosine residues in this region were methylated in the mutant plants. The methylation of the repeats in the *fwa-2* mutant and three revertant alleles of *FWA* was also analyzed and found to be completely absent, as in *fwa-1*.

Methylation has been associated with repression of gene expression and gene silencing in *Arabidopsis* (Jacobsen and Meyerowitz, 1997; Jacobsen, 1999; Kooter et al., 1999; Jacobsen et al., 2000). Therefore, we conclude that *fwa* mutants carry epi alleles of *FWA* and that the dense CG methylation of the repeated sequences is associated with the prevention of *FWA* expression in wild-type plants.

A Late Flowering *ddm1* Line Contains an *FWA* Epi Mutation

DNA of the *ddm1* mutant was shown to be hypomethylated throughout the genome (Vongs et al., 1993). In the progeny of this mutant, stable dominant late flowering lines were observed after several generations. These late flowering traits were genetically mapped to the same position as *FWA* and named *fts* (Kakutani, 1997). To find whether *FWA* might be the cause of the abnormal flowering in these lines, we studied the expression and methylation of *FWA* in early and late flowering *ddm1* lines. Northern blot hybridization showed the presence of *FWA* expression in a late flowering *ddm1* line, whereas no expression could be detected in early flowering lines (Figure 4C). The methylation level of the *FWA* repeated sequences in different early and late flowering *ddm1* lines was analyzed by bisulfite sequencing. Because the *ddm1* mutant was obtained in the Col genetic background, this genotype was also analyzed. The repeated sequences of Col wild type were found to be as densely methylated as in L*er*. A similar level of methylation was found in early flowering *ddm1* lines. However, in a late flowering *ddm1* line these sequences were not methylated, as in the *fwa* mutants. Therefore, we suggest that



seq	% meth
CG	88.6
CA	13.9
CT	12.8
CC	11.7
CYG	19.9
CYY	11.8

Y = ATC

Figure 6. Methylation Pattern of the *FWA* Direct Repeats in L*er* Wild Type

Ten bottom strands and eight top strand clones were sequenced. Rows represent methylation status of individual clones (clone numbers are noted to the right side of the sequence). Filled boxes indicate a 5-methylcytosine in the respective clone, while open boxes denote an unmethylated cytosine residue. The direct repeats are underlined, and numbering of the sequence is relative to the translational start site. The gray shaded GCGC sequences indicate restriction sites for the CfoI restriction enzyme. The table shows percentages of methylated cytosines within different sequence contexts, calculated from the first methylated cytosine to the last.

the late flowering of lines derived from the *ddm1* background is caused by an *FWA* epi mutation.

The Late Flowering Behavior of Plants Transformed with *FWA* Is Unstable

The late flowering phenotype of the *fwa* mutants is very stable, since a screen among 4000 plants of the *fwa-1* marker line for spontaneous early flowering plants did not yield any revertant. In contrast, the flowering behavior of *FWA* transgenic plants was rather unstable. Transgenic L*er* wild-type plants transformed with either of the two cosmids, WS20 and WS28, were analyzed for their flowering behavior through four subsequent generations. Only a small portion of these T1 plants showed a delay in flowering time. None flowered as late as the *fwa* mutant, probably because they are hemizygous for the insert (Figure 7A). Late flowering plants were never observed in the progeny of early transgenic plants. However, the progeny of late flowering T1 plants segregated for flowering time and included plants that flowered as late as the *fwa* mutant (Figure 7A). In all cases tested, the segregation for flowering time did not fit Mendelian ratios for either one or multiple copies of the cosmid. An excess of early flowering plants was observed through T2, T3, and T4 generations. In contrast, Mendelian ratios were observed for the segregation of the cosmid insert in all tested families. This indicates that the distorted segregation of flowering time was not due to reduced transmission of chromosomes bearing the transgene.

We analyzed the expression of *FWA* in different T2 populations. As shown in Figure 7B, the transcript could only be detected in the T2 populations that were segregating late flowering plants and not in the T2 populations that only contained early flowering plants. Thus, *FWA* expression correlated with the flowering phenotype.

In addition, *fwa* mutant plants were transformed with cosmids WS20 and WS28. Surprisingly, several T1 plants that flowered as early as L*er* wild-type were obtained (4 out of 23 plants for WS20 and 17 out of 44 plants for WS28). As shown in Figure 7C, expression of *FWA* was only detected in the late flowering T2 populations and not in the early flowering populations. This indicated that presence of the transgene induced silencing of the endogenous copy of *FWA*.

To test whether *FWA* silencing and the loss of late flowering might be caused by de novo methylation of the *FWA* repeats, we looked at the methylation of several cytosines by Southern blot analysis. This assay was used on *fwa-1* transformed plants because, in this case, *FWA* silencing was induced on both transgene and endogene copies. Genomic DNA was extracted from whole plants of T2 populations and digested with the restriction enzyme CfoI that cuts twice in the repeats and is sensitive for methylation (Figure 6). Late flowering T2 *fwa-1* populations show the same pattern as *fwa-1* itself (Figure 7D). However, T2 populations derived from T1 plants flowering at the same time as wild-type L*er* showed both the L*er* wild-type and *fwa* mutant fragments and two other additional fragments, presumably due to methylation of only one of the two CfoI sites. Therefore, silencing of the *FWA* gene correlates with the presence of methylation in the *FWA* repeated sequences.

Discussion

FWA Encodes a Homeodomain-Containing Transcription Factor

We have identified the *FWA* gene by positional cloning, revealing that it encodes a protein that belongs to the HD-GL2 family (Figure 3B), which is a subclass of plant HD-ZIP homeodomain proteins. Several arguments indicate that *fwa* mutants carry gain-of-function alleles, while *fwa-1R* revertants are loss-of-function mutants of this gene. First, in *fwa* mutants, the flowering delay correlated with overexpression of this gene, compared to wild-type plants. Second, a similar correlation was found in transgenic plants that carry an additional copy of *FWA*. Third, mutations in the *FWA* DNA sequence of *fwa-1R* revertants suppress the late flowering phenotype of the *fwa-1* mutant.

Homeodomain proteins are transcription factors that play an important role in the regulation of developmental decisions through cell fate specification in both animal and plant development. It has been shown that the homeodomain can bind to DNA in a sequence-specific manner and activates or represses the transcription of specific target genes. The leucine zipper can form a dimer that is required for this DNA binding. In addition, the START domain can bind to lipids, which suggests that HD-GL2 proteins function in a lipid-dependent manner (Ponting and Aravind, 1999). The only two genes of this family with a known function are *GLABRA2* (*GL2*), which plays a role in specification of trichome-producing and root hair–developing cells (Rerie et al., 1994; Di Cristina et al., 1996), and *ANL2*, which is involved in anthocyanin distribution and root development (Kubo et al., 1999). The *fwa* mutants are characterized by a delay in flowering initiation, and thus, HD-GL2 proteins also appear to be involved in cell fate changes that occur during transition from the vegetative to the reproductive meristem.

fwa Is a Gain-of-Function Epi Mutant

FWA overexpression in *fwa* mutants indicates that they are gain-of-function alleles of this gene. Although none of the *fwa* alleles have mutations in the DNA sequence of *FWA*, they show a complete absence of cytosine methylation in two direct repeated sequences located in its 5′ promoter and coding regions. This is opposite to the strong methylation observed in wild-type *FWA*. Methylation of coding regions has been shown to lead to a reduction of gene activity in plant cells (Hohn et al., 1996; Jacobsen et al., 2000). Therefore, we concluded that *fwa* mutants are gain-of-function epi alleles of the *FWA* gene in which hypomethylation activates expression and leads to late flowering. Interestingly, overexpression of the most homologous gene to *FWA* (*ANL2*) by activation tagging also resulted in a late flowering phenotype (Weigel et al., 2000). This delay in flowering could be an indirect consequence produced by dominant-negative interference with the function of flowering time genes. In recent years, several loss-of-function epigenetic mutations have been found and studied in plants. As shown for *SUPERMAN* (*SUP*) and *AGAMOUS* (*AG*) epi alleles in *Arabidopsis* (Jacobsen and Meyerowitz, 1997; Jacobsen et al., 2000) and a naturally occurring epi allele of the *Lcyc* gene in *Linaria vulgaris*

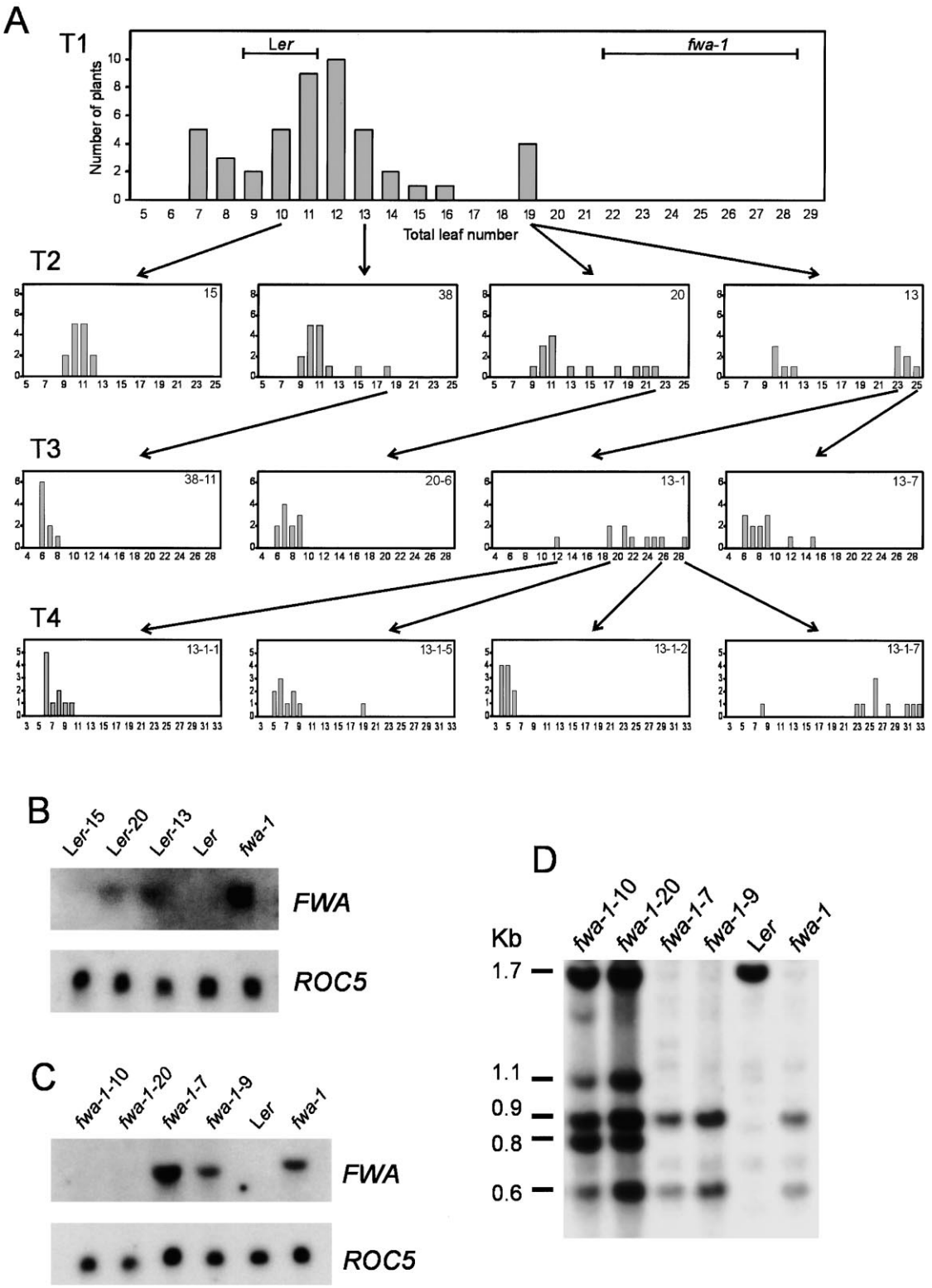


Figure 7. Flowering Time, *FWA* Expression, and Methylation Pattern of L*er* and *fwa-1* Plants Transformed with the *FWA*-Containing Cosmid WS20

(A) Frequency distribution of the number of leaves in the T1 transformants obtained after transformation of L*er* wild-type plants with cosmid WS20 and some of the subsequent T2, T3, and T4 populations. All the T1 plants contained the insert, and all T2, T3, and T4 populations were either homozygous or segregating for the WS20 insertion. The y axis indicates the number of plants, and the x axis indicates the total number of leaves produced by the plant. The ranges of variation for leaf number of L*er* wild-type and *fwa-1* under the growth conditions of this experiment are indicated as horizontal bars.

(Cubas et al., 1999), these mutations are characterized by extensive methylation of a gene, leading to silencing in the mutant. In contrast to these epi mutants where the wild-type allele is expressed, *fwa* mutants provide an example of an epigenetic mechanism that leads to ectopic expression and gain-of-function of an otherwise silenced gene.

FWA messenger was only detected in developing and germinating seeds of wild-type plants, indicating that *FWA* expression is regulated through development. The mechanism by which this occurs remains unknown. It is possible that changes in methylation of the repeated sequences in the 5′ region of *FWA* are involved. These repeats contain both promoter and transcribed regions, and two silencing mechanisms can be speculated: transcriptional gene silencing (TGS), characterized by methylation of promoter regions, and posttranscriptional gene silencing (PTGS), associated with methylation of transcribed regions (Kooter et al., 1999). Typical for PTGS is reactivation of the silenced genes at the onset of each generation (Depicker and Van Montagu, 1997; Kooter et al., 1999), which we observed for *FWA* expression. Furthermore, methylation at nonsymmetrical sites as we found in the *FWA* repeats is characteristic for RNA-directed DNA methylation (RdDM), which can be part of PTGS (Pélissier et al., 1999). In RdDM, RNA elements located in the coding region of a certain mRNA could induce heavy methylation of the corresponding genomic region (Jones et al., 1999). The direct repeat present in the *FWA* mRNA is a candidate for such an RNA element. PTGS has only been observed in the silencing of transgenes thus far. However, it has been speculated to constitute a form of gene regulation that is important for plant growth and development (Depicker and Van Montagu, 1997). Another possibility is that methylation does not affect gene expression equally in all tissues throughout development. Perhaps methylation of the repeats cannot prevent expression of *FWA* during seed development and germination. Consistent with this, the CfoI sites in the *FWA* repeats were methylated in 4-day-old imbibed seeds that showed expression of *FWA* (data not shown). In this respect, it should be noted that plants with a *SUP* epi mutation have a wild-type *SUP* phenotype and expression in ovules, even though the gene is silenced in other tissues (H. Sakai et al., unpublished observations).

fwa Is Locally Defective in DNA Methylation

The *fwa* mutants are characterized by stable hypomethylation of the *FWA* direct repeated sequences and surrounding sequences. This hypomethylation must have been caused during the mutagenesis experiments that yielded these mutants. Several hypotheses could explain this.

The *FWA* hypomethylation might have originated by a wide swath of demethylation of chromosome 4, as a direct consequence of the mutagenesis. In this light, it is interesting to note that one of the *SUPERMAN* hypermethylated epi alleles (*clk-1*) was found in the same plants that contained *fwa*-1. Therefore, this mutagenized plant might have shown disruptions in genomic methylation due to genomic shock caused by EMS.

Secondly, a *ddm1* or *ddm1*-like mutation might have occurred in the mutagenesis experiment, which induced hypomethylation of *FWA* repeats and late flowering. Hypomethylation in *ddm1* is spread over the whole genome, including the 180 bp centromere repeats, rDNA, and the retrotransposon TA3 (Kakutani et al., 1999), which all have a wild-type methylation pattern in *fwa* mutants. During backcrosses with wild type, the original mutation and hypomethylation outside the *FWA* region could have been eliminated from *fwa* mutant plants. In agreement with this, hypomethylation of sequences that are segregated away from the *ddm1* mutation is very stable (Kakutani et al., 1999).

Finally, a mutation in a region containing *cis*-acting local information for methylation might be closely linked to *fwa* and could have caused hypomethylation. In this respect, the characteristics of *fwa* are very similar to those of the human neurogenetic disorders called the Angelman and Prader-Wili syndromes. In patients with these syndromes, chromosome region 15q11-q13 shows abnormal DNA methylation and gene expression in about 2 Mb. Deletions in a region that contains an imprinting center or switch element have been suggested as the cause (Buiting et al., 1995). It has also been shown that a fragment from this region can function as a silencer in transgenic flies, suggesting a link between genomic imprinting and an evolutionary conserved silencing mechanism (Lyko et al., 1998). It could be possible that an element, similar to an imprinting center, is linked to *FWA* and essential for proper methylation in this region. A mutation in this center could explain the local nature of the hypomethylation in *fwa*.

Unlike the *fwa* mutant alleles, *FWA* transgene expression and phenotype are not stably maintained. Interestingly, the late flowering trait is lost after a few generations (Figures 7A and 7B). Furthermore, silencing of both the *FWA* transgenes and endogenes was observed in *fwa* plants that are transformed with constructs containing the *FWA* gene, as shown by the early flowering *fwa* transformants. These plants showed a correlation between silencing of *FWA* and methylation of the repeats (Figures 7C and 7D). Therefore, we suggest that this is at least partly caused by a de novo methylation

(B) Northern blot analysis of *FWA* expression in different T2 bulked populations of L*er* plants, transformed with cosmid WS20. As shown in A, the T2 population L*er*-15 was early flowering, and L*er*-20 and L*er*-13 contained both early and late flowering plants. Plants were grown under LD conditions, and total RNA was extracted 3 weeks after planting. The blot was probed with a fragment of the *ROC5* gene as a control for loading.

(C) Northern blot analysis of *FWA* expression in different T2 bulked populations of *fwa-1* plants, transformed with cosmid WS20. The T2 populations *fwa-1*-10 and *fwa-1*-20 were early flowering, and *fwa-1*-7 and *fwa-1*-9 were late flowering. Plants were grown under LD conditions, and total RNA was extracted 3 weeks after planting. The blot was probed with a fragment of the *ROC5* gene as a control for loading.

(D) Southern blot analysis of the methylation pattern for the same T2 populations analyzed in (C). Genomic DNA was digested with CfoI. A 0.95 Kb probe that completely covered the genomic region, containing the two direct repeats, was used for hybridization. Three DNA fragments of 0.9, 0.6, and 0.2 (not visible) kb were present in the *fwa-1* mutant, and one fragment of 1.7 kb was present in L*er*.

of the repeats. These findings are similar to those reported for the inverted repeats found in the *PAI1-PAI4* gene, which triggers methylation of previously unmethylated *PAI* endogenes after introduction into the plant (Luff et al., 1999). It is likely that the presence of multiple copies (from both the endogene and the transgene) induces homology dependent gene silencing (HDGS) in the transformants (Kooter et al., 1999). Since this silencing occurs in *fwa* transgenic plants, the transgene-dependent silencing mechanism is able to overcome the factor that causes the *fwa* hypomethylation.

The Transition from the Vegetative to the Reproductive Phase Is Mediated by Expression of *FWA*

Increased expression of *FWA* in the *fwa* mutants leads to late flowering. Therefore, *FWA* either represses flowering or promotes vegetative development in these mutants. The molecular mechanism through which this repression occurs is still unknown. Genetic analyses have placed *FWA* in the epistatic group of genes that promote flowering through the photoperiod promotion pathway. In particular, *FWA* appeared fully epistatic to *FT*, since the double mutant *fwa ft* does not flower later than the single mutants (Koornneef et al., 1998a). In addition, double mutants *fwa ap1* and *ft ap1* have a strongly delayed floral initiation, while the double mutants *fwa lfy* and *ft lfy* completely lack flower-like structures (Ruiz-Garcia et al., 1997). Constitutive expression of *LFY* cannot substitute for the late flowering of *ft* and *fwa*, and these mutants in their turn do not interfere with promoter activity of *LFY* as other late flowering mutants do (Nilsson et al., 1998). Thus, it has been speculated that *FT* and *FWA* have similar roles. They control not only the transition to flowering but also floral meristem identity through a common pathway parallel to *LFY* action. The expression pattern of *FT* in an *fwa* mutant background and in wild-type plants is similar, suggesting that *FWA* functions downstream of *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999). However, we could not detect altered expression of *FWA* in the *ft* mutant or in any of the other late flowering mutants. These results suggest that although the *FWA* and *FT* products might work in a common target, their expression is independent of each other.

The loss-of-function mutations of *FWA* (revertant alleles) did not show a flowering phenotype, which makes it unlikely that *FWA* has a function in flowering of wild-type plants. However, it is possible that *FWA* only functions under specific environmental conditions or external stresses in which plants benefit from late flowering. Such conditions might induce hypomethylation of the repeats, enabling expression of *FWA*. In this way silenced genes may act as a reserve of activatable genes, relevant for plant adaptation.

The availability of the *FWA* gene should improve our understanding of its true role in the control of flowering initiation and clarify the significance of the methylated repeats for gene regulation in the near future.

Experimental Procedures

Plant Material

Both *fwa* mutants are in a L*er* background; the *fwa-1* mutant was identified after treatment with EMS and the *fwa-2* mutant after fast neutron irradiation (Koornneef et al., 1991). The L*er* marker line containing the mutations *cer2-1*, *ga5-1*, *fwa-1*, and *abi1-1* was constructed by crossing lines carrying these mutations and selection in subsequent generations.

The *ddm1* mutant lines are in a Col background. The late lines obtained after repeated self-pollination of *ddm1* lines were described by Kakutani (1997).

Growth Conditions and Measurement of Flowering Time

Plants were grown either in a greenhouse with LD light regime (at least 14 hr day length) or in a climate chamber with SD light conditions (8 hr of light per day) as described in Koornneef et al. (1995).

Flowering time was measured by counting the total number of leaves, excluding the cotyledons, since there is a close correlation between leaf number and flowering time (Koornneef et al., 1991).

Construction of the YAC and Cosmid Contigs

YAC clones were obtained from C. Dean (John Innes Centre, Norwich, UK) and analyzed by hybridization with RFLP markers sep2B, CC128 (from C. Dean), GA5 (from J. Zeevaart, Michigan State University, East Lansing, MI, USA), pcr28, pcr34, pcr41, and pcr23 (from J. Giraudat and J. Leung, CNRS, Gif-sur-Yvette, France).

A genomic DNA *fwa-1* library of 27,262 clones with inserts of 15–20 kb was constructed using the binary cosmid vector pCLD 04541, which carries the Agrobacterium LB, RB sequences, and a 35S-NPTII fusion (supplied by C. Dean and C. Lister). The YAC clone EG1F12 was gel purified and hybridized to filters of this library. A cosmid contig was constructed by hybridization of the positive cosmids with YAC's EW18E4, yUP10B5, and yUP11F11 and by hybridizing the cosmids with themselves.

Transformation of Arabidopsis

Selected cosmids for plant transformation were transferred from *Escherichia coli* to *Agrobacterium tumefaciens* (AGLO strain; Lazo et al., 1991) by electroporation. Plants were transformed using the vacuum infiltration transformation procedure (Bechtold et al., 1993). Seeds obtained after infiltration were sterilized for 15 min with 20% bleach in absolute ethanol solution after which they were rinsed two times in absolute ethanol and dried overnight in a flow cabinet. Seeds were sown on plates with selective medium (1× Murashige & Skoog salts, 1% sucrose, 40 μg/ml kanamycin, 0.8% agar [pH 5.8]). The plates were kept in the cold room (4°C) for 4 days and then transferred to a growth room (16 hr of light, 25°C). After 10 days, resistant seedlings were transferred to soil.

DNA and RNA Detection by Gel Blot Hybridization

DNA was isolated from plants, grown in the greenhouse, following basically the protocol of Bernatzky and Tanksley (1986). RNA was isolated from plants grown in the greenhouse or climate chamber, following the protocol of Puissant and Houdebine (1990). Three micrograms of genomic DNA was used for Southern blot analysis, and 25 μg of total RNA was used for Northern blot analysis. Southern and Northern blot analyses were performed, following the protocol supplied with the Hybond-N nylon membranes (Amersham Pharmacia, Uppsala, Sweden). *FWA* expression was detected with a 1.1 kb probe, corresponding to exons 4–8 of the *FWA* gene. A 0.57 kb probe, corresponding to the constitutively expressed cyclophilin gene *ROC5* (Chou and Gasser, 1997) was used as a positive control on Northern blots.

Detection of mRNA by RT–PCR

RNA for RT–PCR was isolated with the Rneasy plant mini kit from Qiagen (Chatsworth, CA). For first-strand cDNA synthesis, 5–10 μg of total RNA was used, and cDNA synthesis was primed by using the standard $dT_{12\text{-}18}$ adaptor primer. The product of the first-strand synthesis reaction was then used for PCR with the primers FWA-E6/7 (5′-GCTCACTCCAACAGATTCAAGCAG-3′), located at the junction of the sixth and seventh exon of the *FWA* gene, and FWA-R2 (5′-GTTGGTAGATGAAAGGGTCGAGAG-3′), located in the eighth exon, which yielded a 0.35 kb fragment. For the control reaction, a fragment of the constitutively expressed *UBIQUITIN10* mRNA (Callis et al., 1995) was amplified, using the primers UBQ10F1 (5′-GATCTTT GCCGGAAAACAATTGGAGGATGGT-3′) and UBQ10R1 (5′-CGACTT

GTCATTAGAAAGAAAGAGATAACAGG-3′), which yielded a 0.5 kb fragment.

Isolation of Complete FWA cDNA by 5′ and 3′ RACE

Ten micrograms of total RNA was used for first-strand cDNA synthesis. The cDNA was amplified and sequenced in three parts. The 5′ part after dCTP tailing with the primers anchor (5′-AAATGGATCCTT CTAGATGCGGGGGGGGGGGGGGGG-3′), adaptor (5′-AAATGG ATCCTTCTAGATGC-3′), and the gene-specific primers fwa-5-1 (5′-ATCTGTCATGCTCTTCTCTA-3′) and fwa-5-2 (5′-TACATTCTCA AGGTGGTCAT-3′). The middle part was amplified with the primers FWA-F2 (5′-ACAGAGGTACGAGCTTGGACAAAG-3′) and FWA-R2. The 3′ part was amplified with the 3′ RACE system kit from GIBCO–BRL (Rockville, MD, USA), using adaptor and amplification primers from the kit and the gene-specific primer fwa-3-1 (5′-ACTCTACCAG CCTTTGATTGGC-3′).

Bisulfite Sequencing

Genomic DNA, isolated from vegetative plants of each genotype was cleaved with DdeI and DraI restriction enzymes. DNA was then treated with sodium bisulfite, amplified, cloned, and sequenced as previously described (Jacobsen et al., 2000). Clones were derived from PCR products of bisulfite-treated DNA using the Invitrogen Original TA Cloning Kit. Several sets of PCR primers were used to amplify the direct repeats and regions outside these repeats.

Acknowledgments

The authors thank Caroline Dean and Clare Lister for their help in the construction of the genomic cosmid library of *fwa-1* and Hildo Offenberg for his help with the RACE PCR. Furthermore they acknowledge Saskia Folmer, Jurre Koning, Leónie Bentsink, and Danny Tholen for technical assistance and Greenomics for sequencing of the *FWA* alleles. Finally, we would like to thank Caroline Dean, Jérôme Giraudat, Jeff Leung, and Jan Zeevaart for the probes that they made available. W. J. J. S. was supported by the European Commission Biotechnology Program DG XII (contract BIO4-CT97-2340) and S. E. J. and J. P. J. by National Institutes of Health grant GM60398.

Received June 29, 2000; revised August 14, 2000.

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GenBank Accession Number

The GenBank accession numbers of the genomic and cDNA sequences of *FWA* are, respectively, AF178688 and AF243535.