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

Wim J. J. Soppe,* Steven E. Jacobsen,† Carlos Alonso-Blanco,*‡ James P. Jackson,‡ Tetsu Kakutani,† Maarten Koornneef,*§ and Anton J. M. Peeters*#

*Laboratory of Genetics
Wageningen University
Dreijenlaan 2
6703 HA Wageningen
The Netherlands
†Department of MCD Biology
University of California, Los Angeles
Los Angeles, California 90095
‡National Institute of Genetics
Yata 1111
Mishima, Shizuoka 411-8540
Japan

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 decrease in DNA methylation (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 (Bam 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 hypomethylated 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
were

marker line carrying the mutations fwa-1 that show a wild-type-like phenotype. Seeds of the open reading frame of this gene; fwa

tions is due to gain-of-function of these alleles, we at- revertant alleles to look for mutations. The three re-

flowering time. Five early flowering plants were obtained tion (Figure 3A). However, the sequences of wild-type

erecta later than Landsberg fwa

tions flower inter-

by ethyl methanesulphonate (EMS) and fwa-2. Characterization of FWA

late flowering pheno-

fwa-1R2

result in loss-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 Ler, 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 thali-

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.

Molecular Cell

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 (Ler) 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 semidomi-

nient (Figure 1).

To determine whether the dominance of fwa muta-

tions is due to gain-of-function of these alleles, we at-

tempted 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 Ler 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.

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 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 Ler 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 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 Ler 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.

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 pattern of FWA. Transcripts were present throughout 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.

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**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.
cases, the same methylation pattern was observed in early flowering Ta3 (Konieczny et al., 1991; Kakutani et al., 1999). In all methylated as in LrDNA (Ronemus et al., 1996), and the retrotransposon sequences of Col wild type were found to be as densely bp centromere repeats (MartõÂnez-Zapater et al., 1986), ground, this genotype was also analyzed. The repeated FWAmutant and fwa-1R revertant alleles, indicating a loss of methylation.

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 methyla-
tion level between fwa mutants and wild type. Hypo-
methylation 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 methyla-
tion 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 al-
lele is normally methylated and the mutant fwa allele is hypomethylated (Figure 5). The genomic DNA methyla-
tion status was also analyzed in repeated sequence regions outside the FWA locus using probes for the 180 bp centromere repeats (Martinez-Zapater et al., 1986), rDNA (Ronemus et al., 1996), and the retrotransposon Ta3 (Konieczny et al., 1991; Kutkaneti 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 meth-
ylation seems to be variable with little preference for sequence context. Analysis of five top- and three bot-
tom-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 (Ja-
cobsen and Meyerowitz, 1997; Jacobsen, 1999; Kooter et al., 1999; Jacobsen et al., 2000). Therefore, we con-
clude 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 hypomethyl-
ated 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 posi-
tion as FWA and named fts (Kakutani, 1997). To find whether FWA might be the cause of the abnormal flow-
ering in these lines, we studied the expression and methy-
lation 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 back-
ground, this genotype was also analyzed. The repeated sequences of Col wild type were found to be as densely methylated as in Ler. A similar level of methylation was found in early flowering ddm1 lines. However, in a late flowering ddm1 line these sequences were not methyl-
ated, as in the fwa mutants. Therefore, we suggest that
Figure 6. Methylation Pattern of the FWA Direct Repeats in Ler 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.

<table>
<thead>
<tr>
<th>seq</th>
<th>% meth</th>
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<tbody>
<tr>
<td>CG</td>
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<tr>
<td>CYY</td>
<td>11.8</td>
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<td>Y = ATC</td>
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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 Ler 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 cosmids. 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 cosmids 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 Ler 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 Ler showed both the Ler 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 epi 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 Ler 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 Ler 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 Ler 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 (Pelissier 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 (ckl-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 retrotranspon 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-Willi 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
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 lty and ft lty 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 Ler 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 Ler 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 SSS-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; Lazaro 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 twice 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 the protocol of Bernatzky and Tanksley (1986). DNA was isolated from plants grown in the greenhouse or climate chamber, following the protocol of Puisant and Houdébin (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 from R. pneazy 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 dT20–adapter primer. The product of the first-strand synthesis reaction was then used for PCR with the primers FWA-E67 (5′-GCTCACTTCAACAGATCTAAGC-3′), located at the junction of the sixth and seventh exon of the FWA gene, and FWA-R2 (5′-GTGGTTGATAGTGAAGGGTCCAGAG-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′-GATCTTTGCCGGAAAACAAATTGGAGGATGGT-3′) and UBQ10R1 (5′-CGACTT
GTACATAGAAAGAGATACAGTGGG-3')

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'-AACTGAGATCTTCTAGTGGGAGGCTGGGGGC-3'), adaptor (5'-AACTGAGATCTCTTGAGCTATGC-3') and the gene-specific primers fwa-5-1 (5'-ATCTGTCATGCTTCTGCTCTCTA-3') and fwa-5-2 (5'-TACATTCTCAAGGGTTGTAC-3'). The middle part was amplified with the primers FWA-F2 (5'-ACAGAGTACGACGCTTGGACAAAG-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'-ATCTGATCCACCTCTTTGATG-3').

Bisulfite Sequencing

Genomic DNA, isolated from vegetative plants of each genotype was cleaved with Ddel and Dral 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.

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References


GenBank Accession Number

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