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Control of *FWA* gene silencing in *Arabidopsis thaliana* by SINE-related direct repeats

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Summary

A unique feature of late-flowering *fwa* epigenetic mutations is that the phenotype is caused by ectopic expression of the homeobox gene *FWA*. During normal development the *FWA* gene is expressed specifically in the endosperm in an imprinted manner. Ectopic *FWA* expression and disruption of imprinting can be induced in mutants of a CG methyltransferase *MET1* (*methyltransferase 1*) or a chromatin-remodeling gene *DDM1* (*decrease in DNA methylation 1*), suggesting that the proper *FWA* expression depends on cytosine methylation. However, critical methylated residues controlling *FWA* silencing are not pinpointed. Nor is it understood how the *FWA* gene is initially methylated and silenced in wild-type plants. Here we mapped sequences critical for *FWA* silencing by application of RdDM (RNA-directed DNA methylation) to a *ddm1*-induced stable *fwa* epiallele. Transcription of double-stranded RNA corresponding to the tandem direct repeats around the *FWA* transcription start site induced *de novo* DNA methylation, transcriptional suppression and phenotypic reversion. The induced changes were heritable even without the transgene, which correlates with inheritance of CG methylation in the direct repeats. The newly silenced *FWA* allele was transcribed in an endosperm-specific and imprinted manner, as is the case for the wild-type *FWA* gene. The results indicate that methylation of the direct repeats, which presumably originated from a short interspersed nuclear element (SINE), is sufficient to induce proper epigenetic control of the *FWA* gene.

Keywords: DNA methylation, epigenetic, transposon, imprinting, RNA-directed DNA methylation.

Introduction

DNA methylation is one of the major heritable epigenetic marks that mediate silencing of sequences such as transposable elements, developmentally regulated genes and imprinted genes (Chan *et al.*, 2005; Rangwala and Richards, 2004; Scott and Spielman, 2004). Most of the imprinted genes in mouse are under control of DNA methylation, as their imprinting is abolished by mutations in DNA methyltransferases (Kaneda *et al.*, 2004; Li *et al.*, 1993). DNA methylation is also involved in imprinting in flowering plants. In Arabidopsis two imprinted genes, *MEDEA* and *FWA*, have been identified (Grossniklaus *et al.*, 1998; Kinoshita *et al.*, 1999, 2004; Soppe *et al.*, 2000; Vielle-Calzada *et al.*, 1999). Their silencing is affected by mutations in the maintenance DNA methyltransferase *MET1* (*METHYLTRANSFERASE 1*) or in the chromatin-remodeling gene *DDM1* (*decrease in DNA methylation 1*; Kinoshita *et al.*, 2004; Vielle-Calzada *et al.*, 1999; Xiao *et al.*, 2003; T. Kinoshita and T. Kakutani, unpublished data), indicating that DNA methylation is also involved in imprinting of these genes. The silent state is default for these genes, and the imprinting is controlled by a 'one-way' activation that depends on the DNA demethylase gene *DEMETER* (Choi *et al.*, 2002; Gehring *et al.*, 2006; Kinoshita *et al.*, 2004).

The approach using DNA-methylation mutants revealed that changes in DNA methylation affect imprinting. However, the critical methylated elements mediating imprinting have not been identified for any of the imprinted Arabidopsis genes (Spillane *et al.*, 2004). Identification of such critical elements would help explain how the methylation-dependent imprinting systems evolved convergently in plants and mammals. In addition, the element identification is essential to understand the mechanism controlling

the plant-specific gene activation by DEMETER demethylase.

The *FWA* gene was originally identified as the gene responsible for the late-flowering phenotype of the *fwa-1* and *fwa-2* mutants (Koornneef *et al.*, 1991). Genetic and molecular analysis revealed that the late-flowering phenotype was not caused by a change in nucleotide sequence, but was due to ectopic expression of the *FWA* gene in the background of reduced DNA methylation (Kakutani, 1997; Soppe *et al.*, 2000). In wild-type plants, although the *FWA* gene is silent in adult tissue, it is specifically expressed in endosperm (Kinoshita *et al.*, 2004). In both the epigenetic mutants and endosperm, a correlation was found between *FWA* transcription and loss of methylation in tandem repeats around the transcription start site (Kinoshita *et al.*, 2004; Soppe *et al.*, 2000). However, there has been no evidence that methylation of this specific region is causing the *FWA* silencing. The *met1* as well as *ddm1* mutations affect methylation across the genome. Not only the tandem repeats, but also large chromosomal regions around them, which can be several megabases long, were hypomethylated in the *met1*, *ddm1* and epigenetic *fwa* mutants (Soppe *et al.*, 2000).

A transgene containing the upstream region of *FWA* often mimics the endogenous *FWA* copy in expression pattern (Figure S1; Kinoshita *et al.*, 2004). Similarly, extra copies of *FWA* become silenced after *Agrobacterium*-mediated transformation (Soppe *et al.*, 2000). Silencing of the *FWA* transgene depends on the *de novo* methyltransferase *DRM*, RNA polymerase IV subunit (*SDE4/NRPD1a*), and genes related to small RNA-processing machinery such as *RNA DEPENDENT RNA POLYMERASE 2*, *DICER-LIKE3*, and *ARGONAUTE4* (Cao and Jacobsen, 2002; Chan *et al.*, 2004). As small RNA is generally made from double-stranded RNA, it is mysterious how the *FWA* transgene, which does not contain an inverted repeat, could become a target of silencing mediated by a small RNA.

In this report, using a stable *ddm1*-induced epigenetic *FWA* line and the RNA-dependent DNA methylation (RdDM) strategy (Mette *et al.*, 2000; Wesley *et al.*, 2001), we show directly that the critical *cis*-elements are the tandem direct repeats around the transcription start site. The silent state of *FWA* was inherited even after the RdDM construct was segregated apart. Inheritance of the silent state in these plants is also associated with inheritance of CG methylation in the direct repeats. Epigenetic controls and the biological significance of the direct repeats are discussed.

Results

Stable inheritance of a ddm1*-induced late-flowering trait*

When an inverted repeat sequence was transcribed from a transgene, *de novo* methylation was induced for the same sequence (Mette *et al.*, 2000). We applied such an RNA-directed DNA methylation (RdDM) strategy to examine directly the critical methylated sequence that silences *FWA*.

For the material to be silenced by RdDM, we first generated a stable epigenetic *FWA* line induced by the *ddm1* mutation. Late-flowering traits were frequently induced in self-pollinated progeny of the *ddm1* mutant (Kakutani, 1997; Kakutani *et al.*, 1996). The *ddm1*-induced late-flowering trait co-segregated with the *FWA* locus irrespective of the presence of the wild-type *DDM1* copy (Kakutani, 1997). To make the background reasonably clean, we back-crossed the late-flowering *ddm1* line three times to parental wild-type Columbia plants. The late-flowering trait was inherited in a dominant manner through the back-crossing and subsequent self-pollination

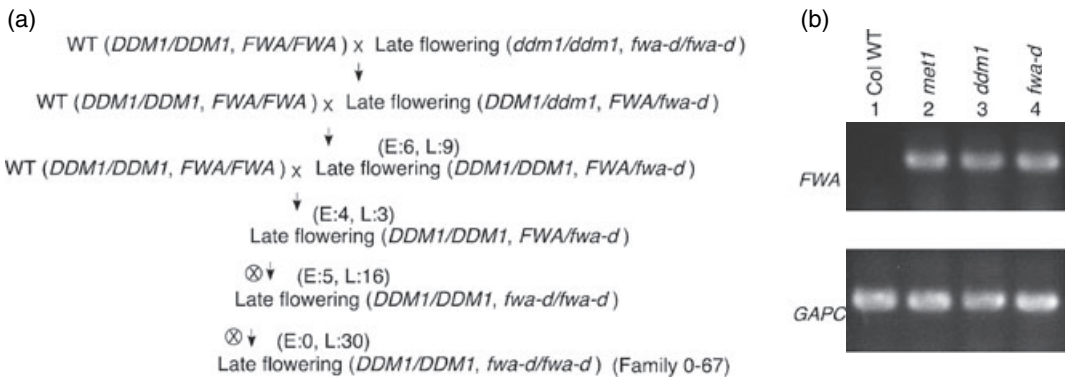

Figure 1. A *ddm1*-induced stable epigenetic late-flowering line.
(a) Back-crossing procedure of a late-flowering *FWA* allele induced by the *ddm1* mutation. The late-flowering epigenetic *FWA* allele (*fwa-d*) was induced by self-pollinating a *ddm1* mutant seven times (Kakutani, 1997; Kakutani *et al.*, 1996). WT, wild-type Columbia plant with wild-type *FWA* and *DDM1* alleles. Segregation of late-flowering (L) and normal flowering (E) plants from each cross is shown by number of plants. The family (0–67) homozygous for the *ddm1*-induced *fwa-d* epiallele was used for the subsequent experiments. X and circled X indicate crossing and self-pollination, respectively.
(b) The late-flowering trait, heritable in the presence of the *DDM1* gene, was associated with ectopic *FWA* expression; *fwa-d* represents self-pollinated progeny of family 0–67 (the last generation in a). Accumulation of *FWA* transcript was detected by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPC) was used as the positive control.

procedures (Figure 1a). This phenotype was maintained even in the presence of the wild-type *DDM1* allele. The late-flowering phenotype, with ectopic *FWA* transcription, was inherited for five generations in the *DDM1*/-background (Figure 1a,b). Further analyses confirmed that the late-flowering trait and the associated ectopic *FWA* expression of this line (*fwa-d*) are stable and genetically linked to the *FWA* locus (Figures S2 and S3).

De novo *silencing of the* FWA *epiallele by a transgene covering direct repeats*

In order to identify the critical methylated *cis*-elements mediating *FWA* silencing, we used the stable epigenetic line (*fwa-d*) and RdDM strategy. For the RdDM procedure, we tested transgenes with inverted repeats covering the 5′ upstream region (construct A, Figure 2a), direct repeats around the transcription start site (construct B), and the first intron region (construct C). Transgenic plants hemizygous for the transgene were crossed to the *ddm1*-induced late-flowering line (*fwa-d* in Figure 1). Phenotypic reversion occurred in plants with the transgene containing the direct repeat around the transcription start site (construct B, Figure 2b). On the other hand, the transgenes containing upstream (construct A) or downstream (construct C) regions did not affect the late-flowering phenotype (Figure 2b). Both the constructs with the upstream and downstream half of construct B (constructs B1 and B2) were effective, although the effects tend to be weaker than the construct covering the entire repeats (Figure 2b, right panel). These observations suggest that methylation around the direct repeats is critical for *FWA* silencing. All the F_1 plants without the transgene remained late flowering (Figure 2), confirming that suppression of the late-flowering phenotype was induced by the transgene. The phenotypic reversion was correlated with silencing of the *FWA* gene (Figure 2c)

Transgene-induced de novo *silencing of the* FWA *epiallele was heritable and associated with CG methylation and small RNA*

In addition to the crossing experiments, we directly transformed the *ddm1*-induced late-flowering line *fwa-d* with the inverted repeat transgenes. The reversion from the late-

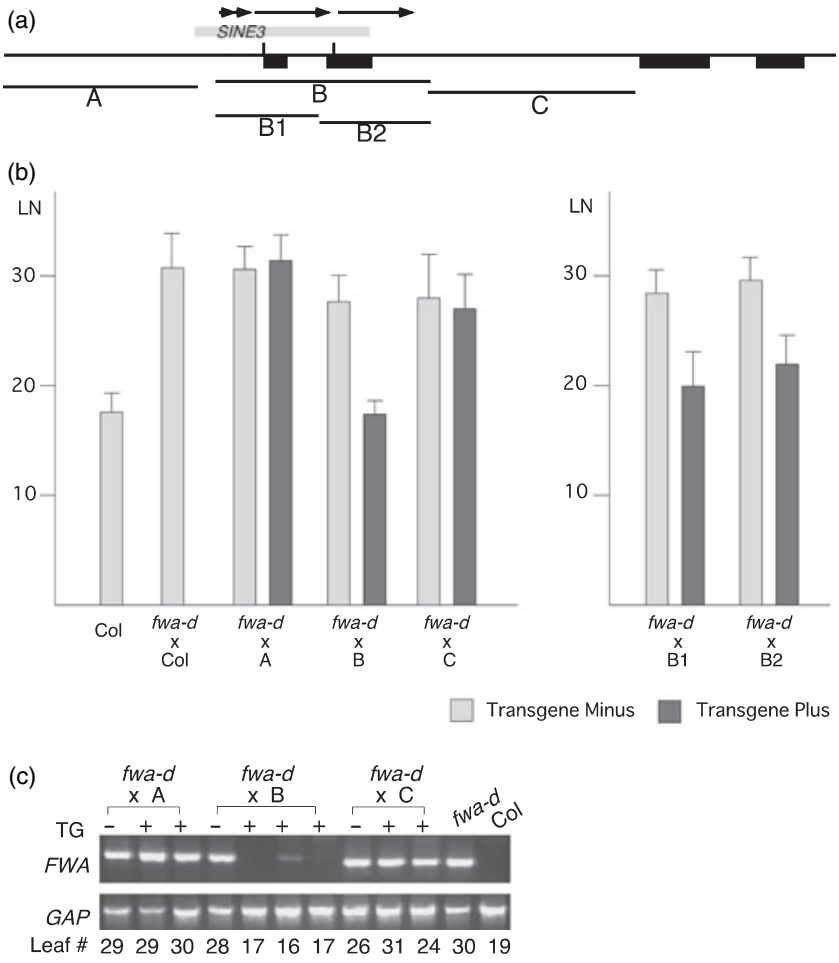

Figure 2. Reversion from the *ddm1*-induced late-flowering trait induced by inverted repeat transgenes.
(a) A, B, C, B1 and B2 indicate regions covered by the inverted-repeat constructs. Black boxes indicate the first four *FWA* exons (Soppe *et al.*, 2000); arrows, direct repeats around the transcription start site; grey bars, regions with characteristics of the *SINE* insertion (Lippman *et al.*, 2004).
(b) The *fwa-d* was crossed to plants hemizygous for the transgenes. Bars represent standard error. Leaf numbers of six to 14 plants were examined for each class with the transgene, while two to nine plants were examined for each class without the transgene.
(c) Transcriptional silencing of *FWA* by the RdDM procedure.

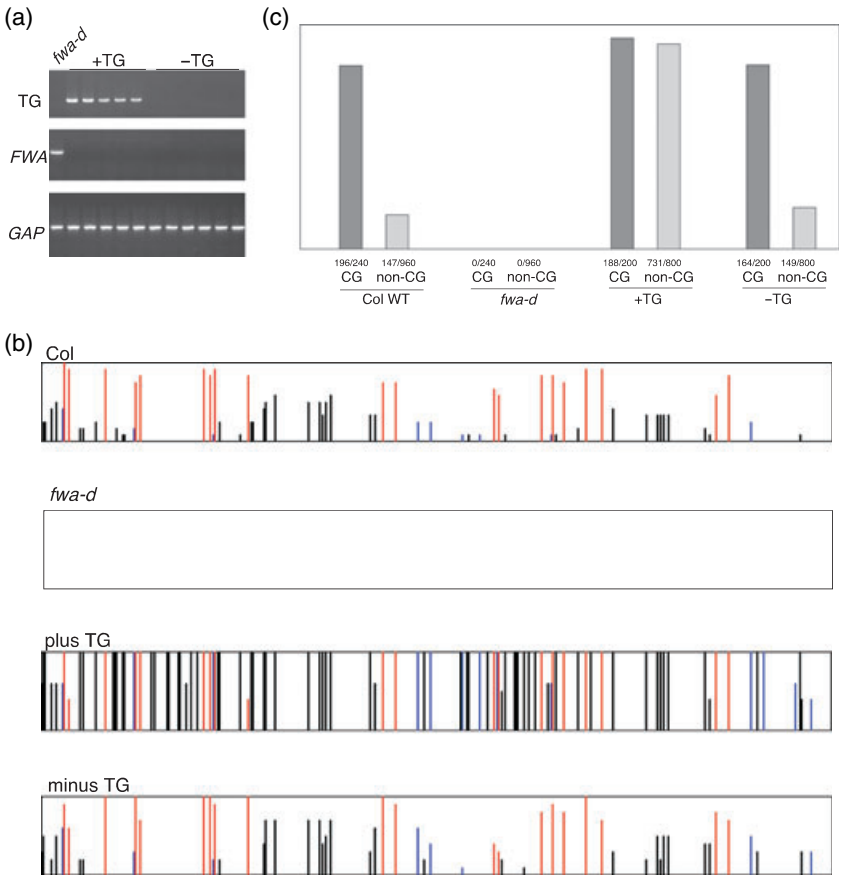

Figure 3. Inheritance of induced transcriptional *FWA* silencing and DNA methylation.
(a) Self-pollinated progeny of a homozygous *fwa-d* line transformed with construct B. Transformation with construct B induced reversion of the late-flowering phenotype. Plants with and without the transgene were selected by PCR (top panel) from the self-pollinated progeny of such a transformant and their *FWA* transcription was analysed by RT-PCR. In both classes, *FWA* transcription remained suppressed, in contrast to the original *fwa-d* line (first lane).
(b) Methylation of the tandem repeats was compared. Ten to 12 clones were examined for each sample; the proportion of methylated clones is shown for each site. Red, blue and black bars represent CG, CNG and asymmetrical sites, respectively.
(c) Proportions of methylation from (b). In plants with the inverted repeat transgene, methylation of both CG and non-CG sites was induced. Methylation, especially in CG sites, was transmitted to the progeny without the transgene, resulting in a methylation pattern similar to that of the wild-type plant. Bisulfite sequencing of the upstream and downstream regions (regions A and C in Figure 2a) revealed that the RdDM transgene covering the direct repeats did not induce spread of methylation (results not shown). In addition, a gene adjacent to *FWA*, which is hypomethylated in the *fwa-d* line, remained hypomethylated in the plant family used here (Figure S5).

flowering phenotype was induced in all four independent lines transformed with construct B. On the other hand, neither construct A or C induced the reversion. Using this system, we examined the inheritance of the silencing effect over generations. In the self-pollinated progeny of a plant with re-silenced *FWA* locus, plants with and without the transgene segregated. Interestingly, the *FWA* gene was silent in both (Figure 3a), suggesting that the effect of the inverted repeat transgene was heritable. These observations suggest that a heritable chromatin-based mechanism, rather than post-transcriptional mRNA degradation, was responsible for the transgene-induced silencing (Hirochika *et al.*, 2000; Vaucheret *et al.*, 1998).

We examined the methylation status of the target regions in those plants by bisulfite sequencing and found that the transcriptional silencing was correlated with *de novo* DNA methylation (Figure 3b,c). In the wild type, cytosines in the direct repeats were methylated mainly in CG sites. On the other hand, both CG and non-CG methylation were induced by the inverted repeat transgene. In the self-pollinated progeny without the transgene, methylation at CG sites was inherited, while at non-CG sites inheritance was less efficient (Figure 3b,c). Still, *FWA* expression was suppressed and an early-flowering phenotype was observed in those plants (Figure 3a). These observations are consistent with the conclusion that CG methylation in the direct repeats is the heritable epigenetic mark required to silence *FWA*.

For the *FWA* direct repeats, a low level of small RNAs has been detected in the wild type (Lippman *et al.*, 2004). A large amount of small RNA was detectable in the *fwa-d* plants with the inverted repeat transgene, which is the expected product from double-stranded RNA (Figure S4, lane 3). After segregating out of the RdDM transgene, the small RNA was detectable at a level comparable with that of the wild type (Figure S4, lane 4). Small RNA has generally been observed in transcriptionally repressed sequences, many of which are structurally related to transposons (Lippman *et al.*, 2004).

After RdDM of direct repeats the fwa *epiallele showed a proper developmental gene-expression pattern*

During normal development in wild-type plants, the *FWA* gene is silent as a default state, and is derepressed in the central cell within the female gametophyte, resulting in imprinted expression in the endosperm (Kinoshita *et al.*, 2004). In order to see whether the *de novo* silencing induced by the double-stranded RNA was derepressed in endosperm, we examined RNA in endosperm. The *FWA* transcript was detected in the endosperm even after *de novo* silencing by the RdDM construct (Figure 4a, left panel). As in the original

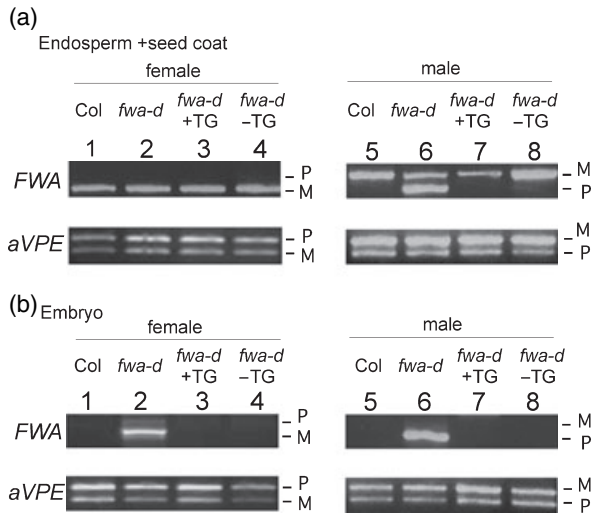

Figure 4. The *FWA* gene silenced by RdDM was transcribed in endosperm in an imprinted manner.
Wild-type Col (lanes 1 and 5), *fwa-d* (lanes 2 and 6), *fwa-d* with the RdDM transgene (lanes 3 and 7) or *fwa-d* after segregating out of the RdDM transgene (lanes 4 and 8) were used as female (left panel) or male (right panel) parents. All are in the Col background and were crossed to wild-type L*er*. In RT-PCR, paternal (P) and maternal (M) transcripts were distinguished by the Col/L*er* polymorphisms as described previously (Kinoshita *et al.*, 2004). RT-PCR was performed using total RNA isolated from dissected embryo (b) or endosperm plus seed-coat fraction (a) from developing seeds at the walking-stick embryo stage. *aVPE* was used as a control with biallelic expression.

wild type, transcription in the endosperm was only from the maternally derived copy; the paternally derived copy remained silent unless it was transmitted directly from the *fwa-d* allele (Figure 4a, right panel). Transcription was not detectable in the embryo unless it was transmitted directly from the *fwa-d* allele (Figure 4b).

Discussion

Using a stable *ddm1*-induced late-flowering line (*fwa-d*) and the RdDM procedure, we determined for which region methylation is critical for *FWA* silencing. *FWA* became silenced when *de novo* methylation was induced in tandem direct repeats around the transcription start site. As in the wild type, the newly silenced *FWA* was transcribed in the endosperm in an imprinted manner. The effect of the RdDM transgene on transcription was heritable, and associated with CG methylation in this region and the presence of corresponding small RNA. These results indicate that methylation of this region is sufficient for proper epigenetic control of the *FWA* gene. The direct-repeat sequence is structurally similar to the short interspersed nuclear element (SINE) retrotransposon (Lippman *et al.*, 2004; Myouga *et al.*, 2001). Transposon sequences can generally be targets of silencing by DNA methylation (Kato *et al.*, 2003; Lippman *et al.*, 2004; Matzke *et al.*, 1999; Miura *et al.*, 2001; Selker *et al.*, 2003; Singer *et al.*, 2001; Yoder *et al.*, 1997). It would be interesting to learn whether the SINE-like sequence *per se* is important, or the repeated feature is necessary for evolution of such an epigenetic control system. In order to understand that, study of the structure and control of the *FWA* repeats in diverse natural accessions or related Arabidopsis species might be informative.

Interestingly, short direct repeats are often associated with mammalian imprinted genes (Neumann *et al.*, 1995). In addition, a number of epigenetic systems use tandem direct repeats as controlling *cis*-elements. A direct repeat was identified as the critical element for control of B paramutation in maize, a classical example of epigenetic silencing heritable over multiple plant generations (Stam *et al.*, 2002). Genomes of higher eukaryotes contain many direct repeats, and their epigenetic controls relate to important cellular processes, such as centromere definition and nucleolar dominance (Grewal and Rice, 2004; Pikaard, 2000). The locus responsible for another *ddm1*-induced epigenetic variant, *BAL*, is composed of a cluster of disease-resistance genes organized as both direct and inverted repeats (Stokes *et al.*, 2002). Some endogenous inverted repeat sequences can be the target of *de novo* methylation (Melquist and Bender, 2003), but the RNAi-based mechanisms might also control silencing of direct repeats (Martienssen, 2003).

The *FWA* epiallele induced by the *ddm1* mutation was stably inherited even in the presence of the wild-type *DDM1* copy (Figures S2 and S3). Inheritance of the *ddm1*-induced epigenetic changes has also been reported in other sequences (Kakutani *et al.*, 1999; Kato *et al.*, 2004; Lippman *et al.*, 2003; Stokes *et al.*, 2002). Not only the *ddm1* but also the *met1* mutation induces a late-flowering phenotype (Kankel *et al.*, 2003; Ronemus *et al.*, 1996; Saze *et al.*, 2003). The *met1*-induced late-flowering trait is also heritable over multiple generations, even after recovery of the *MET1* gene function, although a low-frequency reversion was also reported (Kankel *et al.*, 2003; Ronemus *et al.*, 1996). These observations also suggest that methylation of CG sites is necessary for the maintenance of *FWA* silencing.

In contrast to the inefficient *de novo* silencing in the *ddm1*- or *met1*-induced *FWA* epiallele, tissue-specific and imprinted *FWA* expression can be reproduced in the *FWA-GFP* reporter transgene in the first generation (Figure S1). As the *FWA* transgene in *Agrobacterium* presumably does not have the proper epigenetic marks, such as histone modifications and DNA methylation, this observation suggests that the transgene acquired the epigenetic marks soon after the transformation event. Although *de novo* silencing can be induced by the inverted-repeat RNA, transcription of the *FWA* direct repeat was not sufficient to induce *de novo* silencing. *De novo* silencing of direct repeats may require another unknown factor, which can be mimicked by the *Agrobacterium*-mediated transformation procedure (Chan *et al.*, 2004). A newly integrated DNA may be silenced more

easily than chromatin already having euchromatic marks. Alternatively, double-strand breaks or other types of DNA damage during the integration process may affect the epigenetic state. Recent findings suggest an overlap in the machinery controlling DNA repair and gene silencing (Elmayan *et al.*, 2005; Takeda *et al.*, 2004). In any case, these features of the integrated genes may induce a defence reaction against integration of foreign DNA such as viruses and transposons (Matzke *et al.*, 1999; Yoder *et al.*, 1997).

Experimental procedures

Plant materials

Isolation of *ddm1* mutants from Arabidopsis was as reported by Vongs *et al.* (1993). The *ddm1-1* allele in the Columbia (Col) background was used throughout. The *ddm1-1* mutants and wild-type genotypes were distinguished by examining PCR products with primer pairs 5′-ATTTGCTGATGACCAGGTCCT-3′ and 5′-CATAAACCAATCTCATGAGGC-3′, and restriction digestion by *Nsi*I.

Analysis of RNA and genomic DNA

For *FWA* expression analysis, RNA was prepared using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed using the RETROscript kit (Ambion, St. Austin, TX, USA) or One Step RNA kit (Takara, Ohtsu, Japan). In short, after reverse transcription, cDNA from 50–100-ng input RNA was amplified in 25 PCR cycles and detected by electrophoresis. To detect *FWA* and control GAPC transcript (Shih *et al.*, 1991), primer pairs 5′-GCTCACTCCAACAGATTCAAGCAG-3′ and 5′-GTTGGTAGATGAAAGGGTCGAGAG-3′; and 5′-CACTTGAAGGGTGGTGCCAAG-3′ and 5′-CCTGTTGTCGCCAACGAAGTC-3′, respectively, were used. Products from genomic DNA and mRNA could be distinguished by size as the intron was included within the amplified region. Southern analysis of genomic DNA was performed as described previously (Miura *et al.*, 2004).

Generation of inverted repeat constructs

Transgenes expressing double-stranded RNA were created according to the method of Wesley *et al.* (2001). PCR fragments corresponding to regions A, B, B1, B2 and C (Figure 2a) were amplified from a BAC clone F11K11, which contains the *FWA* gene, with the following primer combinations:

A region: 5′-aaaaagcaggctctctcttcctcatctgcgctta-3′ and 5′-agaaagctgggtcccttaaacaagtaaacactaaaccca-3′;
B region: 5′-aaaaagcaggctctgagttatgggccgaagccca-3′ and 5′-agaaagctgggtctcgggaaccaaaatcattctct-3′;
C region: 5′-aaaaagcaggctcttcccgatttgtctgtttttcgct-3′ and 5′-agaaagctgggtcgcaataacctggacattgcatact-3′
B1 region: 5′-aaaaagcaggctctgagttatgggccgaagccca-3′ and 5′-agaaagctgggtcctgattgtcagtatcccaca-3′;
B2 region: 5′-aaaaagcaggctcttgtgggatactgacaatcag-3′ and 5′-agaaagctgggtctcgggaaccaaaatcattctct-3′.

Each of the primers contains the attB1 or attB2 adapter sequence (underlined) at the 5′ end. To introduce whole attB1 and attB2 adapter sequences to the fragments, all PCR fragments were amplified again using primers 5′-ggggacaagtttgtacaaaaaagcaggct-3′ and 5′-ggggaccactttgtacaagaaagctgggt-3′. The PCR products were then cloned into pHellsgate2 double-stranded RNA producing vector using BP recombinase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Plants were transformed via *Agrobacterium EHA101* by the floral-dip method (Clough and Bent, 1998). Segregation of the pHellsgate transgene (Figure 3a) was examined by PCR using a primer pair, 35Sf: 5′-cgtaagggatgacgcaca-3′ and OCSr: 5′-aggatctgagctacacatgctca-3′.

Detection of DNA methylation by the bisulfite method

Bisulfite sequencing was performed as described by Paulin *et al.* (1998). After the chemical bisulfite reaction, PCR fragments overlapped to regions A–C were amplified with the following primers. For the A region, 5′-aggttytyatyatatayygaaagaatggga-3′ and 5′-ttraaaccatccatrratrrcctartt-3′; B region, 5′-aaagagttatgggyygaag-3′ and 5′-crrraaccaaaatcattctctaaaca-3′; C region, 5′-tgtttagagaatgattttggttyyyg-3′ and 5′-ctaccaacctaarattatttactatttcattccaa-3′. The amplified PCR fragments were gel-purified and cloned into pT7Blue plasmid (Novagen, San Diego, CA, USA), and then 10–12 independent clones were sequenced. The *ASA1* gene (Jeddeloh *et al.*, 1998) was used as a positive control for the bisulfite chemical reaction.

Small RNA Northern analysis

Small RNA was isolated from mature leaves using the mirVana miRNA isolation kit (Ambion). RNA (30 μg) was resolved on denaturing polyacrylamide gels (15%). Electroblotting was performed as described by Llave *et al.* (2002). A radiolabeled probe covering the *FWA* repeat sequence was made by the random priming reaction. Hybridization was performed overnight at 38°C using PerfectHyb Plus buffer (Sigma, St. Louis, MO, USA). Blots were washed at 42°C in 2 × SSC, 0.2% SDS for 10 and 50 min, and in 0.5 × SSC, 0.1% SDS for 60 min.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Proper tissue-specific and imprinted silencing of an *FWA-GFP* transgene.

Figure S2. Stable inheritance of the late flowering trait induced by *ddm1*.

Figure S3. Linkage of the *ddm1*-induced late flowering trait to the *FWA* locus.

Figure S4. siRNA accumulation of the *FWA* tandem repeats.

Figure S5. Inheritance of hypomethylation in a gene adjacent to *FWA*.

Appendix S1. Material and methods and references.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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