Epigenetic transitions leading to heritable, RNA-mediated de novo silencing in *Arabidopsis thaliana*

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Edited by Steven E. Jacobsen, University of California, Los Angeles, CA, and approved November 18, 2014 (received for review July 10, 2014)

In plants, RNA-directed DNA methylation (RdDM), a mechanism where epigenetic modifiers are guided to target loci by small RNAs, plays a major role in silencing of transposable elements (TEs) to maintain genome integrity. So far, two RdDM pathways have been identified: RNA Polymerase IV (PolIV)-RdDM and RNA-dependent RNA Polymerase 6 (RDR6)-RdDM. PolIV-RdDM involves a self-reinforcing feedback mechanism that maintains TE silencing, but cannot explain how epigenetic silencing is first initiated. A function of RDR6-RdDM is to reestablish epigenetic silencing of active TEs, but it is unknown if this pathway can induce DNA methylation at naïve, non-TE loci. To investigate de novo establishment of RdDM, we have used virus-induced gene silencing (VIGS) of an active *FLOWERING WAGENINGEN* epiallele. Using genetic mutants we show that unlike PolIV-RdDM, but like RDR6-RdDM, establishment of VIGS-mediated RdDM requires PolV and DRM2 but not Dicer like-3 and other PolIV pathway components. DNA methylation in VIGS is likely initiated by a process guided by virus-derived small (s) RNAs that are 21/22-nt in length and reinforced or maintained by 24-nt sRNAs. We demonstrate that VIGS-RdDM as a tool for gene silencing can be enhanced by use of mutant plants with increased production of 24-nt sRNAs to reinforce the level of RdDM.

RNA-directed DNA methylation | virus-induced gene silencing | epigenetics | *Arabidopsis thaliana*

Methylation of cytosine (C) residues of DNA is a stable and heritable modification that mediates epigenetic control in eukaryotic genomes. In plants this modification occurs at CG, CHG, and CHH sequences (where H can be C, A, or T) and involves RNA-directed DNA methylation (RdDM) pathways. The DNA methyltransferases in these pathways are guided to target loci by ribonucleoproteins in which a small (s)RNA is the specificity determinant (1, 2).

There are two mechanisms to maintain DNA methylation. In RdDM pathways acting at predominantly transposable elements (TEs), SAWADEE HOMEODOMAIN HOMOLOG (SHH)1 binds and recruits RNA Polymerase IV (PolIV) to transcribe methylated DNA (3, 4). The PolIV transcripts are then made double-stranded by RNA-dependent RNA polymerase (RDR)2 and cleaved by Dicer-like 3 (DCL3) into 24-nt sRNAs that are loaded into and guide AGONAUTE (AGO)4 to complementary scaffold RNAs transcribed from the same locus by RNA polymerase V (PolV). The sRNA-bound AGO and the PolV transcript interact and recruit the de novo methyltransferase DRM2 that modifies C residues of the DNA strand acting as the template for PolV (5).

Maintenance of CG and CHG methylation during DNA replication occurs via MET1, the plant homolog of the mammalian DNA methyltransferase DNMT1, and the plant-specific CHROMOMETHYLASE 3 (CMT3) that works in concert with the SU(VAR)3-9 HOMOLOG (SUVH) SET domain histone methyltransferse KRYPTONITE. Both MET1 and CMT3 act independently of sRNAs but maintenance of CHH methylation within euchromatin is dependent on the continuous operation of the sRNA establishment mechanism, resulting in a self-reinforcing loop (1). This PolIV-RdDM maintenance step is stabilized by SHH1 and the SUVH2/9 SET domain proteins, which bind methylated DNA and continue to recruit PolIV and PolV, respectively to RdDM sites (3, 4, 6, 7). Maintenance of CHH methylation within heterochromatin involves CMT2 and relies on repressive chromatin modifications, independent of sRNAs (8, 9).

The detailed analyses of PolIV-RdDM provide a good explanation of how methylated Cs in an asymmetric context (CHH) can be maintained through feedback mechanisms, but they do not explain how DNA methylation at naïve loci can be first established. A second RdDM pathway has been proposed in which the sRNAs are 21/22-nt rather than 24-nt in length and PolIV is not involved (10–12). This process is referred to as RDR6-RdDM to reflect the identity of the required RDR ortholog in *Arabidopsis*, and it was hypothesized that, in connection with the silencing of TEs and DNA methylation at trans-acting-siRNA (tasiRNA) loci, posttranscriptional gene silencing (PTGS) of PolII-derived transcripts is the trigger. However, the analysis of this mechanism did not conclusively demonstrate a role in RNA-mediated de novo silencing, and the establishment and maintenance phases of silencing at these loci cannot be easily distinguished.

To further investigate the de novo establishment of DNA methylation in plants we infected transgenic plants expressing a GFP transgene with an RNA virus carrying the promoter sequence of the reporter gene (13). On infection, the plant's antiviral defense mechanism produced sRNAs against the virus and the promoter through virus-induced gene silencing (VIGS) (14). The reporter gene was silenced in the infected plant because of increased DNA methylation at its promoter that was maintained in later generations when the virus was no longer present. It was presumed that, like heritable silencing of TEs, the RdDM pathway

Significance

Using virus-induced gene silencing (VIGS) in wild-type and mutant *Arabidopsis*, we characterize a novel mechanism associated with the de novo establishment of heritable epigenetic marks in plants. Once established by this novel mechanism, the epigenetic mark is then reinforced by the previously characterized PolIV pathway of RNA-directed DNA methylation. A similar transition from the novel mechanism to the PolIV pathway is likely to explain many epigenetic phenomena in which RNA-directed DNA methylation is established de novo, including transposon silencing and paramutation. A practical benefit of our work is the identification of a mutant plant genotype in which the maintenance mechanism of epigenetic VIGS is reinforced. This genotype would aid the use of epigenetic VIGS for dissection of gene structure and function.

Author contributions: D.M.B. and D.C.B. designed research; D.M.B. performed research; D.M.B. and D.C.B. analyzed data; and D.M.B. and D.C.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequence reported in this paper has been deposited in the Array Express database, www.ebi.ac.uk/arrayexpress (accession no. E-MTAB-3009).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1413053112/-/DCSupplemental.

was responsible for the establishment of VIGS-RdDM. However, these experiments in *Nicotiana benthamiana* could not test for involvement of pathways that had been previously characterized in *Arabidopsis*.

Using VIGS in *Arabidopsis*, we show that sRNAs can initiate heritable DNA methylation and transcriptional gene silencing (TGS) at an endogenous locus. The virus-derived sRNAs responsible for establishing DNA methylation are likely to be 21/22-nt in length and establishment occurs without canonical PolIV-RdDM, but requires PolV and DRM2. An earlier initiation stage of RNA-mediated de novo silencing may occur independently of DNA methylation. Furthermore, mutant plants producing highly abundant 24-nt sRNAs exhibit reinforced maintenance of RdDM. From our results with VIGS-RdDM, we propose three steps for de novo, heritable DNA methylation at endogenous loci in plants—initiation, establishment, and maintenance—which can occur with or without the presence of 24-nt sRNAs.

Results

VIGS of *FLOWERING WAGENINGEN*. To test the involvement of the PolIV-RdDM pathway in the establishment of DNA methylation, we set up a VIGS system in *Arabidopsis thaliana*. Our strategy was to first characterize VIGS in wild-type plants and then to test RNA silencing mutants for effects on epigenetic silencing of an endogenous target locus. We used tobacco rattle virus (TRV) as the viral vector (15, 16) and an active *FLOWERING WAGENINGEN* (*FWA*) epiallele, in the *fwa-d* epimutant (17), as the target locus. Most of the analyses described here involved plants carrying this epiallele, although for some comparative analyses the plants were wild-type Columbia (Col-0) carrying a naturally silenced allele of *FWA* (18). We refer to the two different epigenotypes as Col-0(FWA^{C}) and Col-0(FWA^{Cme}), respectively, to reflect the status of C methylation at *FWA*.

One TRV construct (TRV:FWAtr) included the direct tandem repeats from the *FWA* promoter and transcribed sequence extending to the second intron within the 5′UTR, a region previously shown to be required for silencing of *FWA* (17, 19). This construct was designed to test epigenetic silencing mechanisms. A second construct, to investigate PTGS, carried part of the *FWA* coding sequence (TRV:FWAcds) (Fig. S1 *A* and *B*).

In the infected plants (V_0 generation) the level of *FWA* sRNAs correlated with the levels of viral RNA (Fig. 1 *A* and *B*) and, with TRV:FWAcds, the *FWA* mRNA was correspondingly less abundant than in control plants. Some repression of *FWA* mRNA was evident in plants infected with TRV:FWAtr, but the level of silencing did not correlate with the level of virus and by 45 d post-infection (dpi) it returned to levels in mock-infected plants (Fig. 1*C* and Fig. S1 *C* and *D*). From these data we conclude that effective posttranscriptional but not epigenetic silencing of *FWA* could be triggered by these TRV VIGS constructs. However, the TRV:FWAcds-infected plants did not flower early (Fig. S1*E*), as would be expected from the reduced expression of *FWA*. This lack of phenotype is most likely because the silencing was too weak or because early flowering was repressed by FWA that had been produced before virus inoculation (20).

The lack of epigenetic VIGS with TRV:FWAtr could indicate that the target promoter sequence was refractory to silencing or that epigenetic silencing was restricted to one allele and was masked by the expressed second allele. To test the second scenario we investigated the flowering time of progeny of TRV:FWAtr-infected plants (V_1 generation) in the expectation that some plants might have inherited two silenced alleles of *FWA*, as in transgene-induced *FWA* silencing (17, 19). Many of the V_1 plants flowered late, as did the Col-0(FWA^{C}) controls. However, in the progeny of TRV:FWAtr- but not TRV- or TRV:FWAcds-infected plants, there were individuals that flowered as early as Col-0(FWA^{Cme}) plants with fully silenced *FWA* (Fig. 1*D* and Fig. S2 *A* and *B*). The proportion of early flowering plants did not follow a simple 1:3 ratio, as would be expected if heterozygous epialleles in the V_0 plant were stably inherited: it was either more or less than 1:3. We conclude that *FWA* silencing affected

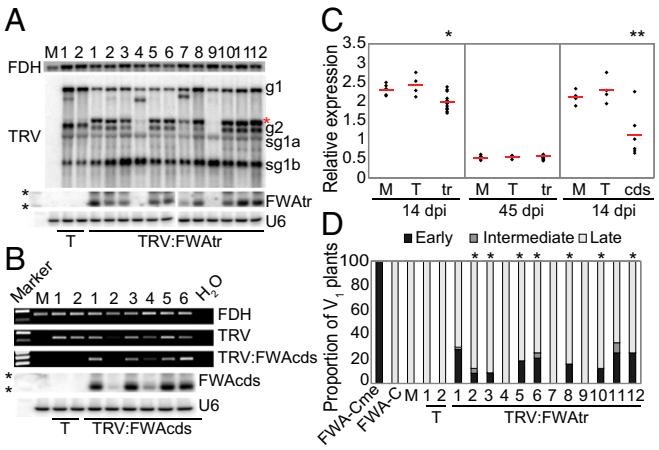

Fig. 1. Establishing VIGS of *FWA*. (*A*, *Upper*) Northern blot with TRV RNA species (Fig. S1*B*) and (*Lower*) sRNA Northern blot with *FWAtr* sRNAs in TRV:FWAtr-, TRV (T)-, or mock (M)-infected Col-0(FWA^{C}) plants, 14 dpi. *FDH* and *U6* were probed as loading controls, respectively. TRV:FWAtr is marked with a red asterisk. The black asterisks represent the 20-nt and 30-nt sRNA markers. For the sRNA Northern blot analysis, TRV:FWAtr-infected samples 7–12 were run on a separate gel as indicated by the white separating line between samples 6 and 7. (*B*, *Upper*) RT-PCR of TRV and TRV:FWAcds in TRV:FWAcds-, TRV (T)-, or mock (M)-infected Col-0(FWA^{C}) plants, 14 dpi. *FDH* was assayed as an internal control. (*Lower*) sRNA Northern blot with *FWAcds* sRNAs in the same plants. *U6* was probed as a loading control. The black asterisks represent the 20-nt and 30-nt sRNA markers. (*C*) *FWA* expression in TRV:FWAtr- (tr), TRV:FWAcds- (cds), TRV- (T), or mock (M)-infected Col-0 (FWA^{C}) plants, 14 dpi and 45 dpi. Each sample is represented by a black diamond and the average of all samples per treatment is represented by the red horizontal line. A two-tailed Student *t* test suggested *FWA* expression was repressed by TRV:FWAtr (*$P < 0.05$) and TRV:FWAcds (**$P < 0.01$), 14dpi. (*D*) Proportion of early- (black), intermediate- (dark gray), and late- (light gray) flowering V_1 progeny from TRV:FWAtr-, TRV (T)-, or mock (M)-infected Col-0(FWA^{C}) plants (*A*), compared with Col-0(FWA^{Cme}) and Col-0(FWA^{C}). Lines with a ratio of <1 (early):3 (late) are marked with a black asterisk.

both alleles but that effects were not seen in the infected plant because they were initially weak and became progressively stronger during the transition between generations. Similar results were obtained with VIGS of the unsilenced *fwa-2* epimutant (18) in the Ler background (Fig. S2*C*).

The progressive silencing of *FWA* was not a result of persistence of TRV:FWAtr because we failed to detect virus in ∼200 tested early flowering progeny using a PCR test (*SI Materials and Methods*). It was likely therefore that silencing was initiated in the V_0 plant without affecting expression of *FWA*. The silencing of this gene would then have been established more completely in the transition between generations, or in the V_1 plants. The proportion of early-flowering V_1 progeny correlated positively with the level of virus infection and *FWAtr* sRNAs in the parent plant, consistent with initiation of epigenetic silencing in the infected plants (compare Fig. 1*A* with Fig. 1*D*).

The early-flowering time in the V_1 progeny of infected plants was associated with changes in DNA methylation at the TRV:FWAtr target site (*FWAtr*). The *FWAtr* DNA in leaf or floral tissue from V_0 plants was not methylated (Fig. S1*F*), but in the V_1 progeny it was hypermethylated at levels similar to those in Col-0(FWA^{Cme}) plants (Fig. 2*A*). Hypermethylation was established in all C sequence contexts (Fig. 2*B*) and, in the earliest flowering V_1 plants, CHH methylation was 10–20% higher than wild-type Col-0(FWA^{Cme}) with naturally silenced *FWA*. In V_1 plants with an intermediate-flowering time the degree of methylation at *FWA* was 20–40% less than in early-flowering plants, although the proportion of methylated C residues in the CHH context was 20–50%: two to five times more than in Col-0 (FWA^{Cme}) (Fig. 2*B* and Fig. S2*D*). Most of this intermediate DNA methylation was at the 5′ end of the TRV:FWAtr target

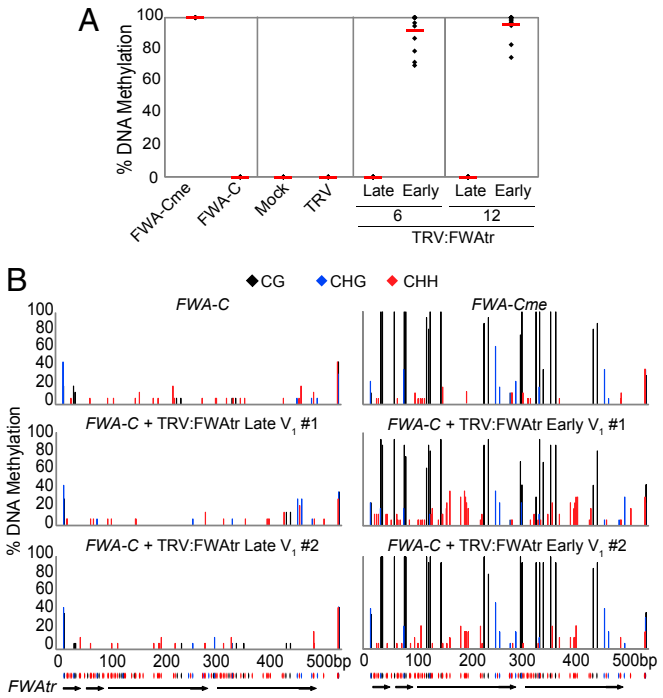

Fig. 2. VIGS-RdDM of *FWA* induces early flowering in V_1 progeny plants. (*A*) DNA methylation (McrBC-qPCR) at *FWAtr* in V_1 progeny of Col-0(FWA^C) plants infected with TRV:FWAtr, TRV, or mock conditions compared with Col-0(FWA^{Cme}) and Col-0(FWA^C) plants. DNA from early- and late-flowering progeny from TRV:FWAtr-infected lines 6 and 12 (Fig. 1*A*) were assayed. Each independent sample is represented by a black diamond and the average of all samples per treatment is represented by the red horizontal line. (*B*) Bisulfite sequencing analysis of DNA methylation at *FWAtr* in early- and late-flowering V_1 progeny plants from *A*. Col-0(FWA^{Cme}) and Col-0(FWA^C) were included as a comparison. The position of C residues along the *FWAtr* region (black arrows) is represented by the ticks on the *x* axis and the context of methylation is represented by the different colors: CG is in black, CHG is in blue, and CHH is in red.

region (Fig. S2*D*), suggesting that VIGS-RdDM was established at the 5′ end of the tandem repeats and that it then spread in the 5′→3′ direction.

Flowering time and DNA methylation at *FWA* cosegregate in a Mendelian ratio in an F_2 population produced from a cross between plants with *FWA* in a silent (FWA^{Cme}) and active (FWA^C) state (18). In contrast, consistent with a progressive increase in *FWA* silencing between generations, the V_2 progeny of intermediate-flowering V_1 plants all gained DNA methylation at *FWA* and flowered at the same time as Col-0(FWA^{Cme}) (Fig. S3 *A* and *B*). This progressive silencing, together with the complete transmission of the *FWA* silenced and hypermethylated phenotype to the V_2 generation from the early-flowering V_1 plants (Fig. S3), indicates that TRV:FWAtr infection leads to transgenerational epigenetic silencing of the *FWA* promoter sequence, as with the original TRV:35S system (13). However, unlike the TRV:35S system, we could not detect the epigenetic silencing in the infected plants (Fig. S1*F*). To explain the *FWA* silencing in the V_1 generation we propose that establishment of the epigenetic mark involved cryptic epigenetic changes to the *FWAtr* sequence in the infected plant. In the following sections we first describe a genetic analysis of *FWA* promoter VIGS and, second, a further analysis of establishment.

Separate RdDM Pathways for CG and CHH Methylation. To investigate the genetic requirements for *FWA* promoter VIGS, we infected FWA^C plants that carried mutations in various genes known to act in PolIV-RdDM (Table S1). Inoculation was with TRV:FWAtr, TRV, or mock conditions (Fig. S4*A*) and, as with the Col-0(FWA^C), there was no change in flowering time of the V_0 plants in any of the mutant backgrounds (Fig. S4*B*).

In the V_1 progeny of infected *poliv*(FWA^C), *rdr2*(FWA^C), *dcl3* (FWA^C), and *ago4*(FWA^C) genotypes, as with Col-0(FWA^C), there were early- and intermediate-flowering plants (Fig. 3*A* and Fig. S5) that gained DNA methylation at *FWAtr* (Fig. 3*B* and Fig. S6*A*). However, the RdDM in all instances with these mutants was predominantly in a CG context and, in the intermediate plants, it was restricted to the 5′ end of the tandem repeat (Fig. S6*B*). In contrast, none of the V_1 progeny of *polv*(FWA^C)- or *drm1/2*(FWA^C)-infected plants were early flowering. From these data we conclude that there are at least two mechanisms involved in RdDM. First, there is a PolIV-, RDR2-, DCL3-, and AGO4-independent process leading to RdDM of C residues in a CG or CHG context. This process is clearly sufficient for silencing of *FWA*. A second process is dependent on PolIV, RDR2, DCL3, AGO4, PolV, and DRM2, and it affects CHH methylation.

In addition to DCL3, *Arabidopsis* encodes DCL1, -2, and -4 that generate 21-nt miRNAs, 22-nt sRNAs, and 21-nt sRNAs, respectively (21) and, in a separate assay for *FWA* silencing, there was a redundant requirement of DCL proteins (22). To further explore the DCL requirement in VIGS-RdDM, we assessed the *dcl2*(FWA^C), *dcl4*(FWA^C), *dcl2/4*(FWA^C), and *dcl2/3/4*(FWA^C) mutants in our assay. The viral load in *dcl2/4*(FWA^C) and *dcl2/3/4*(FWA^C) V_0 plants was much greater than Col-0 (FWA^C) plants (Fig. S4*C*), consistent with the role of DCL2 and DCL4 acting in the antiviral RNA silencing pathway (23). The proportion of early-flowering V_1 progeny from

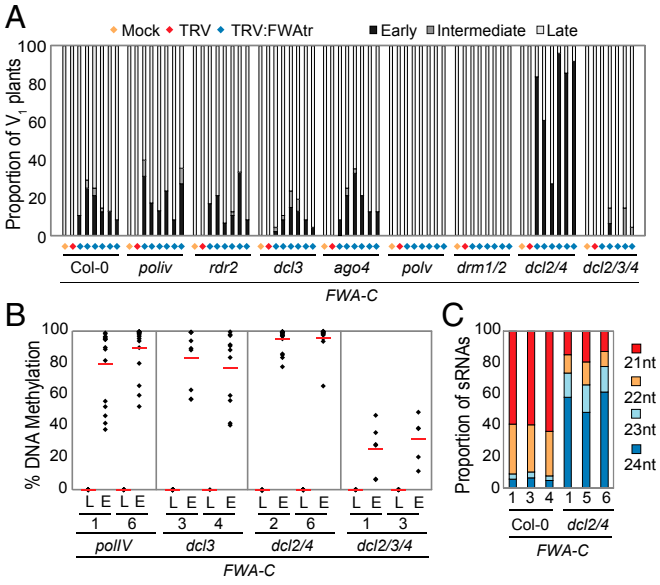

Fig. 3. VIGS-RdDM of *FWA* requires PolV and DRM2 and is enhanced in a *dcl2/4* mutant. (*A*) Proportion of early- (black), intermediate- (dark gray), and late- (light gray) flowering V_1 progeny from Col-0(FWA^C), *poliv*(FWA^C), *rdr2*(FWA^C), *dcl3*(FWA^C), *ago4*(FWA^C), *polv*(FWA^C), *drm1/2*(FWA^C), *dcl2/4* (FWA^C), and *dcl2/3/4*(FWA^C) mutants infected with TRV:FWAtr (blue diamond), TRV (red diamond), or mock (orange diamond) conditions. (*B*) DNA methylation (McrBC-qPCR) at *FWAtr* in early- (E) and late- (L) flowering V_1 progeny from two independent lines of *poliv*(FWA^C), *dcl3*(FWA^C), *dcl2/4* (FWA^C), and *dcl2/3/4*(FWA^C) mutants. Each independent sample is represented by a black diamond and the average of all samples per treatment is represented by the red horizontal line. (*C*) Proportion of different size classes of sRNAs that map to *FWAtr* in Col-0(FWA^C) and *dcl2/4*(FWA^C) TRV:FWAtr-infected V_0 plants. The proportion of 21-nt (red), 22-nt (orange), 23-nt (light blue), and 24-nt (dark blue) sRNA reads was determined from the actual read count, corrected for multiple mapping. Three independent samples per genotype were assessed (Fig. S4 *A* and *C*).

TRV:FWAtr-infected *dcl2*(*FWA*^{C}) and *dcl4*(*FWA*^{C}) plants was similar to Col-0(*FWA*^{C}), but there were many more early-flowering V_1 progeny from *dcl2/4*(*FWA*^{C}) plants (Fig. 3*A* and Fig. S5). For some lines almost 100% of V_1 progeny flowered early and, correspondingly, there was a high degree of DNA methylation at *FWAtr* in these plants (Fig. 3*B* and Fig. S6*A*). This *FWAtr* hypermethylation in *dcl2/4*(*FWA*^{C})-infected plants was more biased to the CG context than with the VIGS of Col-0(*FWA*^{C}) plants.

In the TRV:FWAtr-infected *dcl2/4*(*FWA*^{C}) V_0 plants, unlike the wild-type plants, repression of *FWA* correlated with DNA methylation at *FWAtr* (10–50%) (Fig. S4 *D*–*G*). In a minority of plants, *FWA* was repressed independent of DNA methylation, suggesting PTGS of *FWA* may precede TGS, or an alternative repressive epigenetic mark could be present at *FWA* before initiation of DNA methylation. The pattern of DNA methylation established in the *dcl2/4*(*FWA*^{C}) V_0-infected plants was in all sequence contexts, with increased levels of CHH methylation presumably because of the high level of 24-nt sRNAs in these plants (Fig. S4*A*). However, deep sequencing of sRNAs from TRV:FWAtr-infected *dcl2/4*(*FWA*^{C}) V_0 plants indicated that ~22–35% of sRNAs mapping to the *FWAtr* target region are 21/22-nt in length (compared with 90% in wild-type) (Fig. 3*C* and Fig. S7), consistent with the possibility that 21/22-nt sRNAs play a role in the establishment of DNA methylation in this system.

In contrast, the RdDM and *FWA* silencing in the V_1 progeny of infected *dcl2/3/4*(*FWA*^{C}) plants was not enhanced. TRV:FWAtr infection triggered early or intermediate flowering in a minority of the V_1 generation plants (Fig. 3*A* and Fig. S5) and, overall, the level of DNA methylation, predominantly in the CG context, was less than in the V_1 progeny of Col-0(*FWA*^{C}) plants (Fig. 3*B* and Fig. S6*A*). This finding indicates that the greater silencing of *FWA* in *dcl2/4*(*FWA*^{C}) V_1 plants was dependent on DCL3, and consistent with this interpretation there were abundant 24-nt *FWAtr* sRNAs in the infected *dcl2/4*(*FWA*^{C}) plants (Fig. 3C and Figs. S4*A* and S7). In *dcl2/3/4*(*FWA*^{C}) V_0 plants there were only a low abundance of 21/22-nt *FWAtr* sRNAs (Fig. S4*A*), despite comparable levels of viral load in infected *dcl2/4* (*FWA*^{C}) and *dcl2/3/4*(*FWA*^{C}) mutants (Fig. S4*C*). However, because *FWA* silencing was not eliminated in the V_1 progeny of *dcl2/3/4*(*FWA*^{C}) plants, the epigenetic silencing of *FWA* must have been initiated and established in the absence of DCL3 as with the V_1 progeny of the *dcl3*(*FWA*^{C}) mutant.

Establishment of *FWA* Epigenetic Silencing. To further investigate the establishment step in VIGS-RdDM of *FWA* we made reciprocal crosses between mock-infected and TRV:FWAtr-infected Col-0(*FWA*^{C}) plants (Fig. S8*A*) and analyzed the flowering time of the F_1 progeny. Most of these F_1 plants flowered late, indicating that *FWA* was not silenced. However, F_1 progeny from 15 of 61 lines tested had an intermediate flowering time phenotype (irrespective of the direction of the cross) and had ~50% DNA methylation at *FWAtr* (Fig. 4 and Fig. S8 *B* and *C*). Furthermore, the flowering-time behavior of self-fertilized F_2 populations from the intermediate F_1 plants segregated in a 1:2:1 (early:intermediate:late) ratio (Fig. S8*D*). We conclude from these data that the methylated allele in F_1 plants was inherited from the TRV:FWAtr-infected parent, consistent with the semidominant mutation of *FWA* (18), and that this silent state was not transmitted to the allele from the mock-infected plant. The initiation of *FWA* silencing must have occurred at the DNA or chromatin level in the V_0 plant and these data rule out that a transacting initiating factor, for example an RNA, is transmitted from the infected plant into its progeny.

The early-flowering phenotype was an infrequent characteristic in V_1 progeny of Col-0(*FWA*^{C})-infected plants (185 of 1,061; progeny from 27 TRV:FWAtr-infected lines presented here). However, the *FWA* DNA of all early-flowering V_1 plants was either fully methylated or became fully methylated in the V_2 generation (Fig. S3), suggesting that both gametes had undergone an epigenetic conversion in the V_0 plant that would be manifested as full DNA methylation in the V_1 or V_2 plants. It

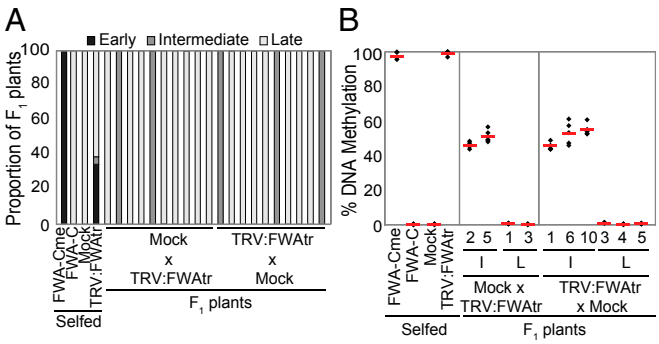

Fig. 4. VIGS-RdDM at *FWA* is established before or during gametogenesis. (*A*) Proportion of early- (black), intermediate- (dark gray), and late- (light gray) flowering F_1 progeny from reciprocal crosses between mock- and TRV:FWAtr-infected Col-0(*FWA*^{C}) plants, compared with self-fertilized Col-0 (*FWA*^{Cme}), Col-0(*FWA*^{C}), mock-, and TRV:FWAtr-infected Col-0(*FWA*^{C}) plants. (*B*) DNA methylation (McrBC-qPCR) at *FWAtr* in intermediate- (I) and late- (L) flowering F_1 progeny from reciprocal crosses between mock- and TRV:FWAtr-infected Col-0(*FWA*^{C}) plants (*A*), compared with self-fertilized control plants. Each independent sample is represented by a black diamond and the average of all samples per treatment is represented by the red horizontal line.

must be, therefore, that the infrequent epigenetic conversion in the infected plant affects either both *FWA* alleles or none. To test this hypothesis, we harvested individual siliques from Col-0 (*FWA*^{C}) V_0 plants infected with TRV:FWAtr (Fig. S8*A*) and measured the flowering time of the individual progeny. We found that most individual siliques produced 100% late-flowering progeny (Fig. S8*E*), consistent with the low frequency of early-flowering behavior transmitted to the V_1 progeny. However, there were some rare siliques with 50–100% of seed that grew into early-flowering plants. The concentration of *FWA* silencing in a few siliques further supports that an epigenetic factor is inherited through both gametes within certain flowers of the infected plant.

To further investigate when silencing of *FWA* is initiated, we assessed the degree of DNA methylation at *FWAtr* in a population of pollen cells from V_0 plants. In Col-0(*FWA*^{Cme}) plants, the *FWA* tandem repeat is demethylated at certain C residues within the vegetative nucleus (VN) of pollen because of the action of the DNA glycosylase DEMETER (DME), but full *FWAtr* DNA methylation is maintained in the sperm cells (SC) (24, 25). Therefore, on average 66% of DNA from pollen of a Col-0 (*FWA*^{Cme}) plant will be fully methylated at *FWAtr*. We predict if *FWA* silencing is initiated in the gametes or earlier, a proportion of pollen SCs from TRV:FWAtr-infected Col-0(*FWA*^{C}) plants will be fully methylated like Col-0(*FWA*^{Cme}).

To test this hypothesis, we bisulfite-sequenced pollen DNA from mock- and TRV:FWAtr-infected Col-0(*FWA*^{C}) (Fig. S8*A*) and *dcl2/4*(*FWA*^{C}) (Fig. S4*D*) plants and compared this to Col-0 (*FWA*^{Cme}) and *dcl2/4*(*FWA*^{Cme}) plants (Fig. 5 and Fig. S9 *A* and *B*). Approximately 20% of clones representing Col-0(*FWA*^{Cme}) and *dcl2/4*(*FWA*^{Cme}) DNA lacked C methylation at residues that are demethylated in the VN (24). This value is consistent with bisulfite sequencing clones representing DNA from SC and the VN, although the proportion of unmethylated DNA is lower than the predicted 33%. The deviation is likely a result of preferential amplification of methylated DNA in the PCR.

No clones of pollen DNA from Col-0(*FWA*^{C}) and *dcl2/4* (*FWA*^{C}) mock-infected plants contained methylated Cs at *FWAtr*. In contrast, 10–25% of clones representing pollen DNA from Col-0 (*FWA*^{C}) TRV:FWAtr-infected plants were methylated in all sequence contexts at the *FWA* target (Fig. 5*A* and Fig. S9*A*). Because of this small sample size, we are unable to differentiate DNA from the SC and VN with confidence, but the presence of fully methylated clones indicates that DNA from SC is represented. Consistent with this interpretation is the correlation of DNA methylation in pollen of TRV:FWAtr-infected Col-0(*FWA*^{C})

plants with the proportion of early-flowering progeny (Fig. S9*C*). This correlation was most striking in samples from *dcl2/4* (*FWA*^{C})-infected plants in which 80–85% of pollen DNA was methylated (Fig. 5*B* and Fig. S9*B*), corresponding to highly abundant early-flowering V_1 progeny (Fig. 3*A* and Fig. S9*D*). From these results we conclude that VIGS-RdDM of *FWA* in TRV:FWAtr-infected plants takes place either at or before gametogenesis.

Discussion

Epigenetic Transitions in de Novo Silencing. Our analysis of *FWA* promoter VIGS was based on the expectation that the canonical PolIV-RdDM pathway would be involved in establishing heritable silencing in the infected plants. In subsequent generations we anticipated that, as with 35S promoter VIGS in *N. benthamiana*, persistence of heritable epigenetic marks would be independent of RNA and dependent on the maintenance methyltransferase, MET1 (13). However, our data are not consistent with that prediction. The data indicate instead that heritable silencing of *FWA* involves a complex sequence of epigenetic transitions in which the canonical PolIV-RdDM pathway is involved but not essential. We propose below that the same sequence of epigenetic transitions applies generally when epigenetic marks in plants are established de novo by an RNA-based mechanism.

Our conclusions are based primarily on two key findings. First there is the unexpected observation that *FWA* promoter VIGS in wild-type plants is largely independent of key components of PolIV-RdDM, including PolIV, RDR2, DCL3, and AGO4 (Fig. 3 and Fig. S6). This finding prompts a radical shift from previous views about RdDM in which most of these proteins were viewed as necessary for establishment of DNA methylation (1, 2).

The second key finding with *FWA* promoter VIGS, unlike 35S promoter VIGS, was that the epigenetic silencing could be established without detectable effect in the V_0 plant and is progressively reinforced over two or three generations. The progression was associated eventually with DNA methylation in CG, CHG, and CHH contexts that spread from the 5′ to the 3′ part of the promoter relative to the direction of transcription (Fig. S2*D*). Consistent with this observation, sRNAs mapped primarily to the 5′ end of *FWAtr* in Col-0(*FWA*^{C}) and *dcl2/4*(*FWA*^{C}) TRV:FWAtr-infected V_0 plants (Fig. S7).

We discuss below how the slow progression of silencing in the *FWA* promoter VIGS system allowed resolution of the epigenetic transitions, including establishment and partially redundant systems for maintenance that is either dependent or independent of RNA. The transitions were less apparent with 35S promoter VIGS because it segued rapidly from establishment in the infected plant into RNA-independent maintenance in the progeny.

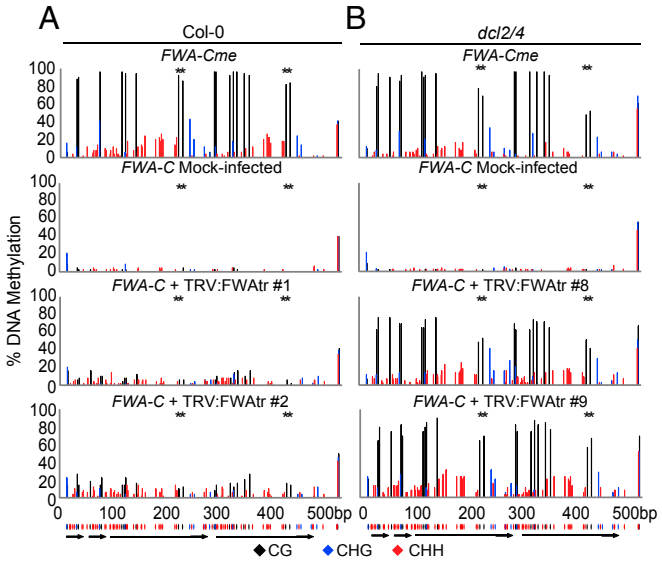

Fig. 5. *FWAtr* is methylated in pollen of TRV:FWAtr-infected plants. Bisulfite sequencing analysis of DNA methylation at *FWAtr* in pollen from mock- and TRV:FWAtr-infected (*A*) Col-0(*FWA*^{C}) and (*B*) *dcl2/4*(*FWA*^{C}) plants. Data for two independent TRV:FWAtr-infected lines per genotype are presented. Col-0(*FWA*^{Cme}) and *dcl2/4*(*FWA*^{Cme}) plants were assayed as a comparison. The position of C residues along the *FWAtr* region is represented by the ticks on the *x* axis and the context of methylation is represented by the different colors: CG is in black, CHG is in blue, and CHH is in red. The four CG residues that are demethylated in the VN by DME (24, 25) are represented by an asterisk.

Initiation and Establishment of Epigenetic Silencing. Mutation of PolV function led to complete loss of VIGS-RdDM (Fig. 3*A* and Figs. S5 and S6*A*) and it is therefore likely that this protein acts in both establishment and RNA-mediated maintenance of silencing. In other RdDM silencing systems, PolV has been implicated in the production of scaffold RNAs that are the binding site of AGO-bound sRNAs that direct DNA methylation to the adjacent chromatin (1, 2). It is anticipated that PolV has a scaffold RNA role in *FWA* promoter VIGS, as proposed for these other RdDM systems. The most abundant sRNAs in the TRV:FWAtr-infected plants are the 21/22-nt size class generated by DCL2, and DCL4 (23) (Figs. 1*A* and 3*C* and Figs. S4*A* and S7), and it is probable, as in the RDR6-RdDM system (12), that these RNAs account for the primary RdDM mediated by VIGS.

Mutation of the de novo methyltransferase DRM2 leads to complete loss of VIGS-RdDM (Fig. 3*A* and Figs. S5 and S6*A*), suggesting that DNA methylation is an early epigenetic mark in the establishment mechanism. DNA methylation at *FWAtr* could be detected in the pollen of plants undergoing VIGS-RdDM, indicating that DNA methylation is established early (Fig. 5 and Fig. S8). However, we could not detect this *FWA* promoter methylation in the vegetative tissue of Col-0(*FWA*^{C}) V_0 plants (Fig. S1*F*), and it remains possible that another process—initiation—precedes establishment of DNA methylation in the gametes. Such an alternative process could involve histone modifications (1) or it could involve persistent RNAs, like those involved in "recovery" from virus infection (26). Recovery is a long-lived and sequence-specific immunity to secondary infection by plant viruses including TRV that is mediated by RNA silencing.

Maintenance of Epigenetic Silencing. Our data indicate that, following establishment of *FWA* promoter VIGS, the epigenetic marks are maintained in the V_1 and subsequent progeny by two partially redundant maintenance mechanisms. An RNA-independent maintenance relies on the recognition of hemimethylated Cs in the symmetrical context of newly replicated DNA motifs by the DNA methyltransferases MET1 and CMT3. In contrast, the RNA-dependent maintenance is sequence motif-independent. It involves canonical PolIV-RdDM in which methyl DNA-binding proteins recruit the 24-nt sRNA biogenesis proteins to the genomic site of primary RdDM. The 24-nt sRNAs would then continue to direct the DNA methyltransferases to the unmethylated strand of newly replicated DNA. DCL3 is a key protein in this process because it generates the 24-nt sRNAs.

The *dcl3* phenotype is consistent with this interpretation because the mutation did not affect establishment but it did reduce the level of CHH methylation (Fig. 3 and Fig. S6). That there was no effect of *dcl3* on the frequency of early-flowering progeny is probably because the overall level of *FWAtr* DNA methylation in the *dcl3* mutant was similar to that observed for the wild-type, although it was more biased to CG and CHG methylation. We propose that initiation and establishment of *FWA* silencing in *dcl3* would have been as in wild-type plants but that maintenance was only via the RNA-independent mechanism.

Conclusion

Our proposed model for *FWA* promoter VIGS involves initiation and establishment mechanisms likely mediated by 21/22-nt sRNAs followed by two maintenance mechanisms that are either

dependent on 24-nt sRNAs or independent of RNA (Fig. S10). This model can account for the high level of DNA methylation at *FWAtr* in all contexts in the V_1 progeny of *dcl2/4* plants and the reduction of this strong silencing in *dcl2/3/4* V_1 progeny to levels of CG methylation that were lower than in the *dcl3* mutant (Fig. 3*B* and Fig. S6). To explain these effects, we propose that initiation and establishment of *FWA* silencing in the *dcl2/4* and *dcl2/3/4* mutants is at a lower level than in wild-type and *dcl3* because the 21/22-nt initiator sRNAs would be produced, presumably, by DCL1 alone rather than DCL1, -2, and -4 in wild-type plants. The low level of primary RdDM in the *dcl2/4* plants would be compensated for by the RNA-dependent maintenance mechanism that would be supercharged by the very abundant 24-nt sRNAs (Fig. 3*C* and Figs. S4*A* and S7).

This model could also explain the transition from PTGS to TGS of the *Evade* retroelement (27), the RDR6-RdDM pathway in which 21/22-nt sRNAs direct DNA methylation at active TEs (12), the 21-nt sRNAs from VN of pollen having a role in epigenetic modification of DNA in the SC (28), and with secondary epigenetically activated siRNAs (easiRNAs) and tasiRNAs that guide RdDM at active TEs and *TAS* genes, respectively (11, 29). The paradigm could also be reconciled with the finding that PolIV is apparently required for de novo RdDM of an *FWA* transgene in plants with an endogenous FWA^{Cme} (30). We propose that, independent of PolIV, there is very low-level silencing of this transgene similar to the establishment of silencing in our V_0 plants (Fig. 5*A*). Transgenes are often prone to spontaneous silencing because they produce aberrant RNA. However, in the presence of PolIV, 24-nt sRNAs would maintain and reinforce the silencing of this transgene. Some of these 24nt sRNAs may have been produced at FWA^{Cme} and acted *in trans* at the transgene locus.

The interpretation that 24-nt sRNAs are not sufficient for establishment is also consistent with the similar levels of 24-nt sRNAs complementary to *FWAtr* in plants with active and silent *FWA*, despite the different levels of DNA methylation at *FWAtr* (19). Similarly, in paramutation, there is evidence that 24-nt sRNAs are not sufficient to transmit silencing from one allele to another (31). It is therefore possible that the requirement for 21/22-nt sRNAs for establishment and the switch to 24-nt sRNAs for maintenance is a general feature of de novo RdDM in plants (12).

An attraction of our model is that it requires only a single process to explain diverse initiation of epigenetic silencing in many systems. However, we have not yet been able to carry out a rigorous test of the model because no mutant devoid of 21/22-nt viral sRNAs is available. In the absence of such a test we concede that a second mechanism for establishment of RdDM involving 24nt sRNAs remains possible.

In addition to mechanistic insights, our data also suggest an enhanced strategy for heritable, epigenetic VIGS of endogenous loci. The target locus should be in a *dcl2/4* mutant to establish gene silencing and there should be a functional DCL3 to ensure maximal reinforcement through the RNA-dependent maintenance mechanism. To boost the system further, a target with a high number of C residues in the CG context will ensure efficiency of the RNA-independent maintenance mechanism. This strategy may have application if epigenetic silencing would be useful for crop plant improvement.

Materials and Methods

Plant Methods. Wild-type [Col-0(FWA^{Cme})], the *fwa-d* epimutant (17) [Col-0 (FWA^{C})] and various RNA silencing mutants in the Col-0 background (Table S1) were grown using standard plant growth methods. See *SI Materials and Methods*.

Viral Inoculations. Previously described TRV VIGS vectors (15, 16) were modified to contain the *FWAtr* or *FWAcds* DNA sequences. Virus replication was carried out according to ref. 15 and young leaves of 3-wk-old *Arabidopsis* plants were mechanically inoculated. See *SI Materials and Methods*.

Nucleic Acid Analyses. Standard protocols for RNA and DNA extraction were performed. See *SI Materials and Methods* for subsequent techniques and Table S2 for oligonucleotides.

ACKNOWLEDGMENTS. We thank Sebastian Müller for bioinformatic support; Shuoya Tang and Mel Steer for technical assistance; and Ian Henderson, Tim Hore, Jake Harris, and Quentin Gouil for critical reading prior to submission. Work in the D.C.B. laboratory is supported by The Gatsby Charitable Foundation, the EU FP7 Collaborative Project Grant AENEAS, and the European Research Council Advanced Investigator Grant ERC-2013-AdG 340642. D.C.B. is the Royal Society Edward Penley Abraham Research Professor.

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