Histone arginine methylation is required for vernalization-induced epigenetic silencing of *FLC* in winter-annual *Arabidopsis thaliana*

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Certain plant varieties typically require prolonged exposure to the cold of winter to become competent to flower rapidly in the spring. This process is known as vernalization. In *Arabidopsis thaliana*, vernalization renders plants competent to flower by epigenetically silencing the strong floral repressor *FLOWERING LOCUS C* (*FLC*). As a result of vernalization, levels of lysine-9 and lysine-27 trimethylation on histone 3, modifications that are characteristic of facultative heterochromatin in plants, increase at *FLC* chromatin. We have identified a mutant, *protein arginine methyltransferase 5* (*atprmt5*), that fails to flower rapidly after vernalization treatment. *AtPRMT5* encodes a type II protein arginine methyltransferase (PRMT) that, in winter-annual strains, is required for epigenetic silencing of *FLC* and for the vernalization-mediated histone modifications characteristic of the vernalized state. Furthermore, the levels of arginine methylation of *FLC* chromatin increase after vernalization. Therefore, arginine methylation of *FLC* chromatin is part of the histone code that is required for mitotic stability of the vernalized state.

Plants have evolved a range of strategies to ensure that flowering occurs at the optimum time of the year for reproductive success. In some plants, a vernalization requirement is a key component of the reproductive strategy. Vernalization is the acquisition of the competence to flower resulting from exposure to the prolonged cold of the winter season (1). A vernalization requirement permits plants to become established during the fall season without the risk of flowering as winter sets in. During the cold of winter, these plants become vernalized, which enables them to flower during the favorable conditions of spring.

A study of natural variation in the vernalization requirement among *Arabidopsis* accessions led to some of the first examples of identifying genes that influence *Arabidopsis* life history traits. Napp-Zinn (2) identified *FRIGIDA* (*FRI*) as a locus that confers a vernalization requirement. *FLOWERING LOCUS* C (*FLC*) was later identified as cooperating with *FRI* to confer a vernalization requirement (3, 4). *FRI* encodes a plant-specific protein of unknown biochemical function (5), and *FLC* encodes a transcriptional regulator that is a repressor of the floral transition. *FRI* acts to maintain *FLC* transcription at levels sufficient to effectively repress flowering before vernalization (6, 7).

Vernalization can be considered an epigenetic phenomenon in the sense that the cold of winter induces a mitotically stable change in gene expression that persists well into the spring season after the inducing signal, cold, is no longer present. During winter, *FLC* chromatin undergoes a transition from an actively transcribed state to a heterochromatin-like state (8, 9). This change in chromatin structure is associated with a decrease in the level of histone modifications characteristic of active chromatin such as trimethylation of lysine-4 on histone 3 (H3K4) and H3 acetylation, and an increase in levels of the repressive modifications characteristic of inactive chromatin such as methylation of lysine-9 on histone 3 (H3K9) and lysine-27 on histone 3 (H3K27) (8–10). These repressive modifications are maintained in the spring and are likely to be part of the stable switch that "remembers" winter. Like many other epigenetic phenomena, the molecular nature of vernalization is consistent with the "histone code hypothesis," which posits that the combination of covalent modifications to histone tails contributes to the creation of stable states of gene activity (11).

In *Arabidopsis*, a number of genes have been identified that are required for vernalization-induced epigenetic silencing of *FLC*. Of these, *VIN3* is the only gene that is transcriptionally activated by prolonged exposure to cold (9). VIN3 can interact with the related protein VIN3-LIKE 1/VERNALIZATION 5 (12, 13) and can exist in an *Arabidopsis* PRC2 (Polycomb Repression Complex)-like complex, which includes VERNALIZATION 2, CURLY LEAF, SWINGER, and FERTILIZATION INDEPENDENT ENDOSPERM (14). In *Drosophila* and other animals, the PRC2 complex establishes methylation of H3K27, thus laying the foundation for the maintenance of stable transcriptional repression by the PRC1 complex (15).

Unlike the situation in animals, plants do not possess PRC1 components; instead, the repression of *FLC* is maintained by LHP1 (LIKE HETEROCHROMATIN PROTEIN 1) (10, 16). Furthermore, *FLC* repression is associated with H3K9 trimethylation as well as H3K27 trimethylation (13). In the *lhp1* mutant, H3K9 methylation occurs at *FLC* chromatin during cold, but after a return to warm conditions H3K9 methylation levels are reduced, indicating that *LHP1* is required to maintain vernalization-induced PRC2-like histone modifications at *FLC* (10). VERNALIZATION 1 is also required for vernalization-mediated H3K9 methylation at *FLC* chromatin (8, 9) and may be part of the system that renders the vernalized state mitotically stable.

Here we discuss the involvement of *AtPRMT5*, a type II protein arginine methyltransferase, in the vernalization-induced epigenetic silencing of *FLC*. *AtPRMT5* was identified in a genetic screen for vernalization-insensitive mutants. In a winter-annual background, *atprmt5* mutants fail to stably repress *FLC* after vernalization treatment, indicating that arginine methylation is required for maintenance of the silenced state. In *atprmt5* mutants, global levels of symmetric dimethylation of arginine 3 of histone 4 (H4R3sme2) are greatly reduced, and this modifi-

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cation is reduced at *FLC* chromatin as well. Furthermore, in *FRI;atprmt5* mutants, levels of H3K9 and H3K27 methylation at *FLC* chromatin do not increase after vernalization. Thus, *AtPRMT5* is required for the repressive histone modifications that are thought to contribute to establishment and maintenance of the vernalized state in *Arabidopsis* winter annuals.

Results

***atprmt5* Mutants Exhibit an Attenuated Vernalization Response.** The Columbia (Col) accession of *Arabidopsis thaliana* contains a naturally occurring lesion in *FRIGIDA* and therefore does not have a strong vernalization requirement (5). Introgression of a functional allele of *FRI* into Col from the vernalization-requiring San Feliu-2 accession of *Arabidopsis* created a "nearly isogenic" Col line (Col *FRI*) that has a strong vernalization requirement and thus behaves as a winter annual (17). We identified a mutant from a genetic screen in Col *FRI* that fails to flower rapidly after a vernalization treatment (Fig. 1 *A* and *B*). The vernalization-insensitive phenotype is recessive, and a single T-DNA locus cosegregates with the mutant phenotype. *Arabidopsis* sequences that flanked the T-DNA insert aligned to the 3′ UTR of At4g31120, which is predicted to encode a protein arginine methyltransferase (Fig. 1*C*) (18). An alignment of the predicted primary amino acid sequence of At4g31120 to protein databases revealed high sequence identity to human PRMT5 (Protein Arginine Methyltransferase 5) (19). An additional At4g31120 mutant was isolated from the Salk T-DNA collection (Fig. 1*C*). This allele also displays a vernalization-insensitive phenotype when present in the Col *FRI* genetic background (Fig. 1*B*). Expression of At4g31120 during a vernalization time course revealed that, unlike *VIN3*, At4g31120 mRNA abundance is not affected by prolonged exposure to cold (Fig. 1*D*).

While our work was in progress, two recent articles reported the phenotype of mutations in *AtPRMT5* in a Col (*fri*) background (20, 21). Those mutants were named *atskb1-1*/*atprmt5-1* and *atskb1-2*/*atprmt5-2*. We hereafter refer to the additional alleles we present in this study as *atprmt5-3* and *atprmt5-4* (Fig. 1*C*).

***AtPRMT5* Is Required to Repress *FLC* in the Absence of Vernalization.** In the absence of vernalization, *atprmt5* mutants in Col *FRI* typically fail to flower after generating well over 120 rosette leaves from the primary meristem. In contrast, the Col *FRI* parental line flowers without vernalization after producing ≈60 rosette leaves in our growth conditions (Fig. 1 *B* and *E*).

atprmt5;flc-3 double mutants were generated to determine whether *FLC* was required for this enhancement of the delayed-flowering phenotype of *atprmt5* in a Col *FRI* background. *atprmt5;flc-3* double mutants flowered at the same time as *flc-3* single mutants, indicating that enhanced *FLC* expression is responsible for the delay in flowering in Col *FRI* (Fig. 1*B*).

***AtPRMT5* Is Required for Maintenance of Vernalization-Induced *FLC* Silencing in Col *FRI*, but Not for Repression of Other Members of the *FLC* Clade.** To determine whether the vernalization-insensitive phenotype of *FRI;atprmt5* was due to a failure to repress *FLC* during and after a vernalizing cold exposure, we evaluated mRNA levels of *FLC* throughout a vernalization time course. In Col *FRI*, *FLC* is expressed at high levels before vernalization and is repressed by prolonged cold treatment (6) (Fig. 2*A*). In *atprmt5* mutants in Col *FRI*, *FLC* is strongly expressed before vernalization and is only partially repressed during cold (Fig. 2*A*). Furthermore, *FLC* repression is not maintained after plants are removed from the cold (Fig. 2*A*). This failure to maintain *FLC* silencing likely contributes to the vernalization-insensitive phenotype of *atprmt5*.

Although, as noted above, *FRI;atprmt5* mutants are later-flowering than the parental Col *FRI* line both with and without vernalization, *FRI;atprmt5* mutant plants do flower more rapidly after a vernalizing cold exposure than nonvernalized mutants (Fig. 1*B*). This "partial" response to vernalization is not likely to be a result of *FLC* repression because stable repression of *FLC* fails to occur in *FRI;atprmt5-3*. Therefore, other floral repressors that are silenced by vernalization are likely contributing to the acceleration of *FRI;atprmt5* flowering after vernalization (Fig. 1*B*).

FLC is a member of a clade of floral repressors that includes *FLM*/*MAF1* and *MAF2-5* (22–24). All members of this clade, except for *MAF5*, are subjected to repression by vernalization (22), and thus vernalization can accelerate flowering in *flc* mutants (25). The effect on flowering due to the vernalization-mediated repression of *FLM*/*MAF1* and certain *MAFs* is most pronounced in short days (9, 25).

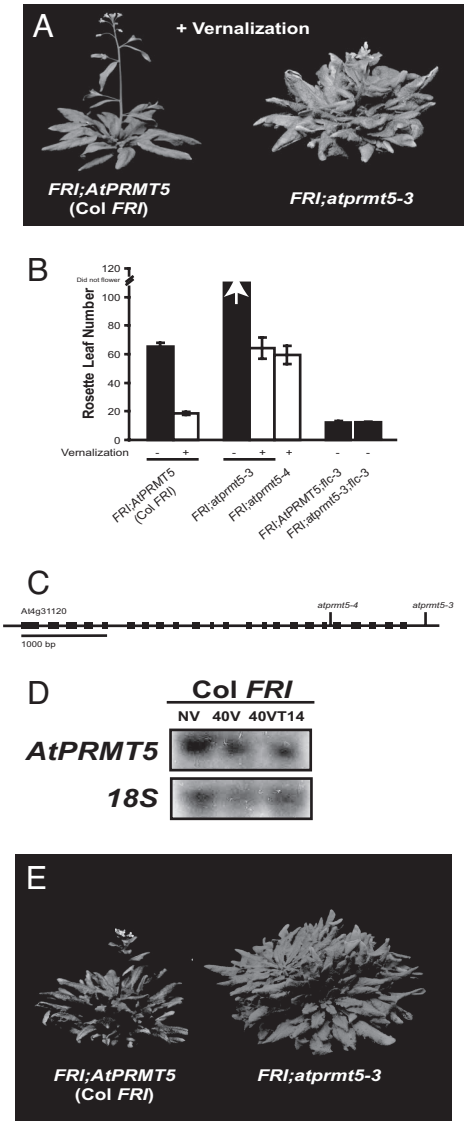

Fig. 1. *FRI;atprmt5* mutants display a vernalization-insensitive phenotype. (*A*) Image of *FRI:AtPRMT5* versus *FRI;atprmt5-3* after 40 days of cold treatment. (*B*) Flowering time represented as total rosette leaf number. (*C*) Gene structure of *AtPRMT5* (At4g31120). Lines indicate location of T-DNA alleles [*atprmt5-3* was isolated in our genetic screen, and *atprmt5-4* was isolated from the Salk collection of T-DNA mutants (accession no. SALK_073624)]. (*D*) Northern blot analysis of *AtPRMT5* during cold treatment. (*E*) Image of Col *FRI* and *FRI;atprmt5*-3 without vernalization treatment. RNA was isolated from seedlings without vernalization (NV), with 40 days of cold treatment (40VT0), and with 40 days of cold treatment followed by 14 days in the warm (40VT14).

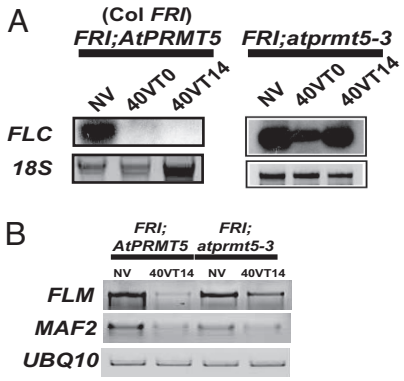

Fig. 2. *atprmt5-3* flowering phenotypes are dependent on *FLC*. (*A*) Northern blot analysis of *FLC* mRNA levels in Col *FRI* and *FRI;atprmt5-3*. *FLC* expression resumes after plants are returned to warm conditions in the *FRI;atprmt5-3* mutant, indicating that *AtPRMT5* is required for vernalization-induced stable repression of *FLC*. (*B*) RT-PCR analysis of mRNA levels of members of the *FLC* clade.

atprmt5;flc-3 double mutants and *flc-3* mutants both flower more rapidly in short days after vernalization [supporting information (SI) Fig. 6], indicating that the partial vernalization response observed in *atprmt5* might be due to repression of members of the *FLC* clade. Indeed, mRNA levels of two members of the *FLC* clade tested (*FLM*/*MAF1* and *MAF2*) are reduced after vernalization in both *FRI;atprmt5* and *fri;atprmt5* when compared with their respective wild types (Fig. 2*B*; data shown for *FRI* backgrounds only). Thus, *AtPRMT5* is required for the vernalization pathway to repress *FLC* but not for the vernalization pathway to repress some other members of the *FLC* clade.

***AtPRMT5* Contributes to *FLC* Repression in the Absence of *FRI*.** *atprmt5* mutants also exhibit a delay in flowering in the wild-type Col genetic background, which lacks a functional *FRI* (Fig. 3 *A* and *B*; we refer to wild-type Col hereafter as Col *fri*). The delay in flowering in Col *fri* was also observed by Pei *et al.* (21) and Wang *et al.* (20). In Col *fri*, *atprmt5-3* and *atprmt5-4* mutants flower after producing ≈45 rosette leaves in contrast to Col, which flowers after generating ≈18 leaves in our growth conditions (Fig. 3*B*; data shown for *atprmt5-3*).

Late flowering and vernalization responsiveness are defining characteristics of autonomous-pathway mutants (26). Autonomous-pathway genes are involved in *FLC* repression, and the delayed-flowering phenotype of autonomous-pathway mutants depends on *FLC* function; i.e., a lesion in *flc* suppresses the delayed flowering of autonomous-pathway mutants (25). *fri;atprmt5* is similar to autonomous-pathway mutants because (*i*) its late-flowering phenotype is associated with elevated levels of *FLC* mRNA (Fig. 3*B*), (*ii*) its late flowering is suppressed by lesions in *flc* (Fig. 1*B*), and (*iii*) in *fri;atprmt5 FLC* is stably repressed by vernalization (Fig. 3*C*). Thus, in a *fri*, but not a *FRI*, background, vernalization is able to cause a stable suppression of *FLC* expression. Interestingly, the vernalization response of *fri;atprmt5* (as measured by total leaf number after cold treatment) is attenuated relative to other autonomous-pathway mutants (Fig. 3*D*). The slightly attenuated vernalization response of *fri;atprmt5* relative to other autonomous-pathway mutants may be to be due to "leaky" *FLC* repression in *fri;atprmt5*.

Global Levels of H4R3sme2 Are Reduced in *atprmt5* Mutants. Arginine methylation is found in three forms: monomethylation, asymmetric dimethylation, and symmetric dimethylation. Of these forms, monomethylation and asymmetric dimethylation

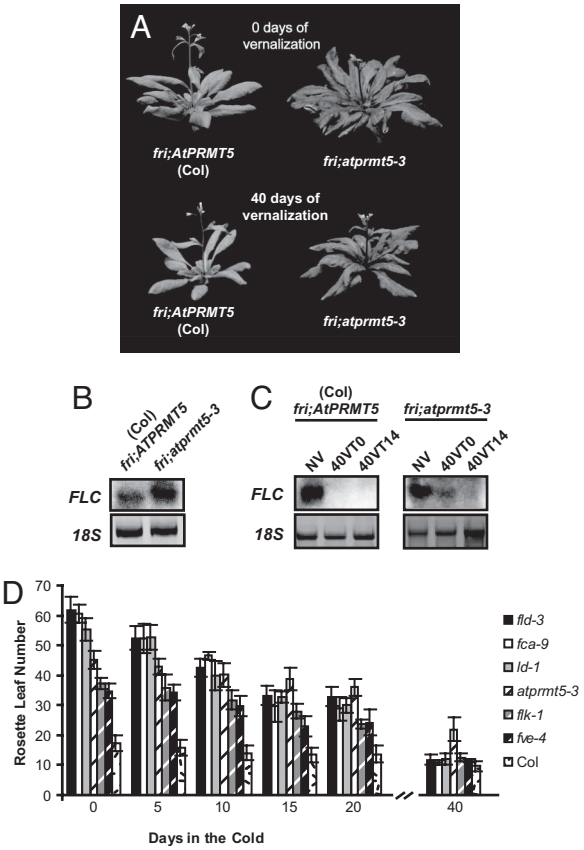

Fig. 3. *AtPRMT5* represses *FLC* before vernalization. (*A*) Image of Col *fri* and *fri;atprmt5-3* before and after 40 days of vernalization. (*B*) Northern blot analysis of *FLC* mRNA expression in Col *fri* and *fri;atprmt5-3*. (*C*) Northern blot analysis of *FLC* mRNA expression in Col *fri* and *fri;atprmt5-3* during a vernalization time course. (*D*) Flowering time, represented by total rosette leaf number, of known autonomous-pathway mutants and *fri;atprmt5-3* with various days of cold treatment as indicated on the *x* axis.

are generally associated with transcriptional activity whereas symmetric dimethylation is associated with transcriptional silencing (27). PRMT5 can symmetrically dimethylate arginine residues of proteins involved in RNA processing (28) and histones, specifically histone 4 (29). *AtPRMT5* appears to be the only type II PRMT encoded in the *Arabidopsis* genome. Analysis of global levels of H4R3sme2 in *fri;atprmt5-3* and *fri;atprmt5-4* compared with Col *fri* revealed that loss of *AtPRMT5* results in a genome-wide reduction in symmetric dimethylation of arginine-3 at histone 4 (H4R3sme2) (Fig. 4*A*; also reported in ref. 20). Furthermore, the difference between the two alleles of *atprmt5* in the degree of H4R3sme2 level reduction correlates well with the strength of each allele predicted by the T-DNA position and the severity of the other associated mutant phenotypes (*atprmt5-4* is more severe than *atprmt5-3* as discussed below).

Levels of H4R3sme2 at *FLC* Chromatin Increase as a Result of Prolonged Exposure to Cold. H4R3sme2 levels at *FLC* increase after a vernalizing cold treatment (Fig. 4*B*). As previously reported (8–10), methylation of H3K9 and H3K27 also increases as a result of prolonged cold treatment, and these two repressive histone modifications may be required, along with *LIKE-HETEROCHROMATIN PROTEIN 1*, for maintenance of the repressed state (10, 16). In *atprmt5* mutants in Col *FRI*, no increase in H3K9 and H3K27 methylation occurs after cold treatment (Fig. 4 *C* and *D*), indicating that, in this winter-annual

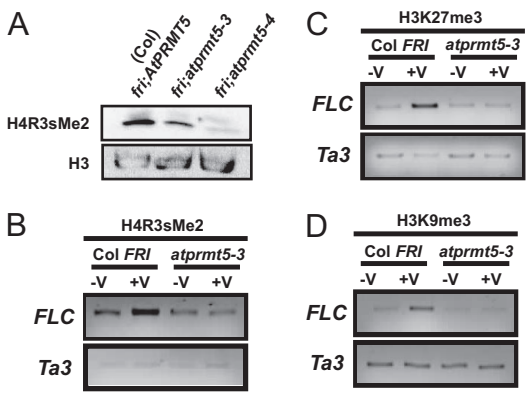

Fig. 4. *AtPRMT5* is required for the epigenetic silencing of *FLC*. (*A*) Immunoblot analysis of symmetric dimethylation of arginine-3 on histone 4 (H4R3sme2) on protein extracts isolated from seedlings. Histone 3 (H3) was used as a loading control. (*B–D*) ChIP analysis of *FLC* chromatin before and after vernalization using antibodies against H4R3sme2 (*B*), H3K27me3 (*C*), and H3K9me3 (*D*). Ta3 was used to standardize our samples, and the *FLC-P* region was analyzed.

background, H4R3sme2 is required for repressive histone modifications such as trimethylation of H3K9 and H2K27 at *FLC* chromatin.

***NDPK2* Is a *FLC*-Independent Target of AtPRMT5.** *atprmt5* mutant plants have darker green leaves with shorter petioles and consequently form a compact rosette. These mutants also exhibit slight downward curling of the leaves, and mutant leaves are wider than wild type (Fig. 5*A*). These phenotypes are stronger in *atprmt5-4* than *atprmt5*-3. Although the flowering phenotype of these alleles is identical, the nonflowering phenotypes are independent of *FLC* (data not shown). The pleiotropy associated with *atprmt5* is not surprising given the global decrease in H4R3sme2 in *atprmt5* mutants. We performed microarray analyses to identify genes that are derepressed in *fri;atprmt5* mutants; such derepressed genes are potential targets of AtPRMT5 and are listed in SI Table 1. One candidate, *NUCLEOTIDE DIPHOSPHATE KINASE 2* (*NDPK2*), exhibited a >3-fold increase of mRNA levels in the mutant compared with wild type. These data were confirmed by RT-PCR analysis of *NDPK2* RNA levels (Fig. 5*B*). Furthermore, *NDPK2* is indeed a locus that contains H4R3sme2; ChIP analysis of *NDPK2* chromatin revealed the presence of H4R3sme2 in wild type compared with the *fri;atprmt5-3* mutant, indicating that symmetric arginine methylation is required for proper levels of *NDPK2* expression (Fig. 5*C*). Last, there is an expansion in leaf width in transgenic lines overexpressing *NDPK2* when compared with the wild-type Wassilewskija accession (Fig. 5*D*). Because a similar expansion in leaf width is also observed in *atprmt5* mutants (Fig. 5*A*), it is likely that the observed leaf expansion phenotype in *atprmt5* mutants is due to the derepression of *NPDK2*. Interestingly, *NM23* (the human homolog of *NDPK2*) is a target of PRMT5 (the human homolog of *AtPRMT5*) (30). It is intriguing that *PRMT5* modifies *NDPK2* chromatin with arginine methylation in both *Arabidopsis* and humans.

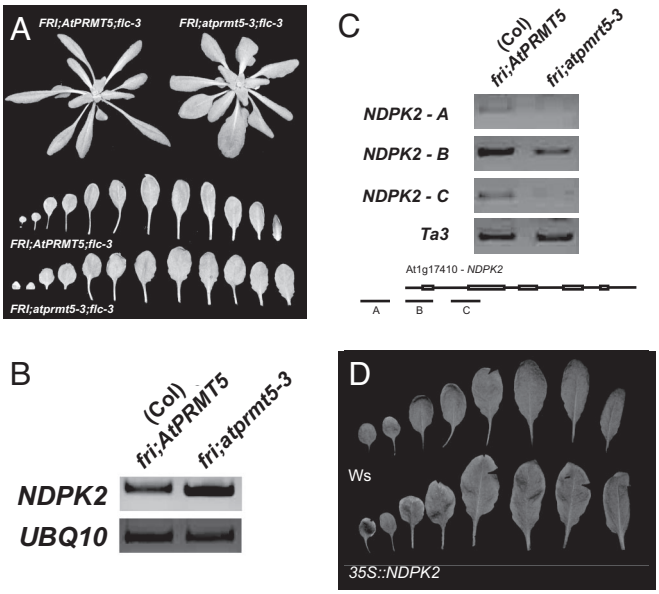

Fig. 5. *NDPK2* represents another target of *AtPRMT5*. (*A*) Image of pleiotropy present in *FRI;atprmt5-3* compared with Col *FRI* (double mutants between *FRI;atprmt5-3* and *FRI;flc-3* still result in pleiotropy). *atprmt5-3* mutants display shorter petioles, a compact rosette, and dark green leaves. (*B*) RT-PCR analysis of *NDPK2* mRNA levels in Col *fri* and *fri;atprmt5-3*. (*C*) ChIP of *NDPK2* chromatin from seedlings. Samples were immunoprecipitated with H4R3sme2 antibodies. (*D*) Overexpression of *NDPK2* in the Wassilewskija (Ws) accession results in plants with some similarity to *atprmt5-3*.

Discussion

AtPRMT5 is required for repression of *FLC* in the absence of vernalization in both *FRI* and *fri* backgrounds, as well as for vernalization-mediated repression of *FLC* in a *FRI* background. In *FRI;atprmt5* there is some degree of *FLC* repression during cold exposure, but *FLC* expression is restored to approximately pre-cold-treated levels after plants are removed from cold. Reduction of expression of *Arabidopsis* PRC2 components *CLF*, *SWN*, and *FIE* results in phenotypes similar to *FRI;atprmt5* such as delayed flowering and an inability to maintain *FLC* repression after vernalization (14). Lesions in *VRN1* and *VRN2* also compromise the maintenance of *FLC* repression (31, 32). Thus, arginine methylation might function in conjunction with polycomb repression machinery to maintain stable repression of target genes.

An *atprmt5* lesion causes a vernalization-insensitive phenotype in combination with the autonomous-pathway mutation *luminidependens-1* (data not shown). Therefore, the vernalization insensitivity of *atprmt5* is not due to a specific interaction of *FRI* with *atprmt5*, but rather is likely due to the combination of *atprmt5* with situations in which *FLC* is highly transcribed.

The effect of *AtPRMT5* on flowering appears to be specific to *FLC*. This is in contrast to *VIN3*, which affects *FLC* and several additional members of the *FLC* clade. One measure of *FLC* clade specificity is the effect of a mutation on the vernalization response in short days. For example, with vernalization, *flc-3* single mutants and *atprmt5;flc-3* double mutants both flower more rapidly in short days, whereas *vin3;flc-3* double mutants do not (9). The "residual" short-day vernalization response of *FRI:atprmt5* or *flc-3* single mutants is likely due to vernalization-mediated repression of other members of the *FLC* clade. The lack of response in *vin3* mutants likely results from a requirement for *VIN3* for repression of most or all *FLC* clade members. The specificity of *AtPRMT5* for *FLC* is not unprecedented: components of both the autonomous pathway and certain suppressors of *FRI*-mediated late flowering can also specifically affect *FLC* transcription without effect on other *FLC* clade members (22, 25, 33, 34).

In the absence of vernalization, loss of *atprmt5* delays flowering in both the winter-annual Col *FRI* and the rapid-flowering Col *fri* strains. In this respect, *AtPRMT5* behaves in a manner similar to genes in the autonomous pathway. Indeed, in a *fri* background, the only difference between *atprmt5* and other

autonomous-pathway mutants is the slight attenuation of the vernalization response (Fig. 3*D*). Interestingly, two autonomous-pathway mutants (*fve* and *flowering locus d*) also exhibited a mild late-flowering phenotype after vernalization when in the *FRI* background, similar to *FRI;atprmt5* (Scott Michaels, personal communication, and our unpublished results). Other autonomous-pathway mutants present in a *FRI* background, such as *fca*, *fpa*, *flowering locus k homology domain*, and *luminidependens*, do not exhibit late-flowering phenotypes after vernalization. These results indicate that within the autonomous pathway there is specialization because a subset of autonomous-pathway genes are also required for vernalization in the presence of *FRI*.

We have shown that vernalization results in an increased level of H4R3sme2 at *FLC* chromatin. Moreover, the delayed-flowering phenotype of nonvernalized *atprmt5* mutants is consistent with a model in which some degree of H4R3sme2 is required for "basal" repression of *FLC*. Indeed, Wang *et al.* (20) demonstrated, by immunoprecipitation, that AtPRMT5 interacts with *FLC* chromatin from nonvernalized plants. Thus, *FLC* chromatin appears to be a substrate of AtPRMT5 in both vernalized and nonvernalized plants. The increased level of H4R3sme2 at *FLC* after vernalization may result from increased AtPRMT5 activity or a reduction of activity that removes H4R3sme2.

Although the simplest model for the repression of *FLC* by AtPRMT5 is a direct methylation of H4R3 at *FLC* chromatin, it should be noted that mammalian PRMT5 associates with a large protein complex, referred to as the methylosome (35), which methylates components of a small nuclear ribonucleoprotein complex involved in splicing of target pre-mRNAs (28). Interestingly, many members of the *Arabidopsis* autonomous pathway contain RNA-binding domains (FCA, FPA, and FLK) that may have a function in splicing (36). Thus, it is possible that some portion of the effect of AtPRMT5 on *FLC* expression could be due to a failure to symmetrically dimethylate arginine residues of proteins involved in mRNA splicing.

AtPRMT5 appears to be a single-copy gene, and it is predicted to encode the only type II protein arginine methyltransferase in *Arabidopsis*. The viability of *atprmt5* mutants may reflect that none of the alleles described in this or other studies (20, 21) are nulls, but a more likely possibility is that loss of *AtPRMT5* activity does not result in a lethal disruption of gene expression. Indeed, lesions in other single-copy proteins involved in chromatin modification such as components of the PAF1 complex in both yeast and *Arabidopsis* are also viable (37, 38).

A definitive role for histone arginine methylation has not yet been established. There is a correlation between active gene expression and H4R3asMe2 (asymmetric dimethylation) and between H4R3sme2 and repressed loci (39, 40), although only a few loci that contain histone arginine methylation have been studied and there are examples to the contrary. Our study is consistent with a role for H4R3sme2 in the epigenetic silencing of *FLC*. Indeed, the vernalization-mediated increase in H4R3sme2 at *FLC* chromatin is the first example of a dynamic change in H4R3sme2 at a specific locus in response to an environmental signal. H4R3sme2 may be necessary to provide a proper chromatin substrate to enable levels of other vernalization-mediated histone modifications such as H3K9 and H3K27 methylation to increase. Alternatively, the increase in H4R3sme2 may occur concurrently with both H3K9 and H3K27 methylation. In either scenario it is clear that H4R3sme2 is required for both methylation of H3K9 and H3K27 and the subsequent epigenetic silencing of *FLC*.

Very few targets of PRMT5 have been reported in any organism. We identified *NDPK2* as a possible target because it was derepressed in *fri;atprmt5* mutants relative to wild-type Col *fri*. ChIP analysis of the *NDPK2* locus revealed the presence of H4R3sme2. Interestingly, in human cell cultures *NM23* (the human homolog of *NDPK2*) is a direct transcriptional target of PRMT5 (the human homolog of *AtPRMT5*) (28). In the absence of human *prmt5*, *NM23* is derepressed, and, conversely, in cell lines overexpressing human PRMT5, *NM23* expression levels are reduced. It is intriguing that *NDPK2* is a target of *PRMT5*-mediated arginine methylation in both *Arabidopsis* and humans. That this PRMT5 target is conserved may be coincidence (i.e., convergent evolution) or an ancient conserved relationship.

Methods

Plant Material. All genotypes used in this work are in the Columbia (Col) background with the exception of the *35S::NDPK2*, which is in the Wassilewskija accession. The *atprmt5-4* insertion line in the Col background was isolated from the Salk collection (http://signal.salk.edu; accession no. SALK_073624) (41).

Growth Conditions. Plants were grown in long days (16 h light/8 h dark) or short days (8 h light/16 h dark) at 22°C under cool-white fluorescent lights (Sylvania) at 100 μmol·m^{-2}·s^{-1}. For experiments involving vernalization, seeds were incubated on agar-solidified medium containing 0.65 g/liter Peters Excel 15-5-15 fertilizer (Grace Sierra) at 4°C for 2 days, transferred to short days at 22°C, allowed to germinate for 5 days, and then transferred to 4°C in short days for the number of days noted in the figure legends.

T-DNA-Flanking Sequence Analysis. The sequence flanking the T-DNA of *atprmt5-3* was obtained by thermal asymmetric interlaced PCR (18). T-DNA borders were defined by sequencing PCR products obtained by using a T-DNA border primer and a gene-specific primer.

RNA Isolation and Blotting. In all cases, total RNA was isolated from 7-day-old seedlings by using TRIzol (Invitrogen) following the manufacturer's protocol. RNA was subsequently purified and concentrated by using an RNeasy column (Qiagen). For RNA blots, 15 μg of total RNA was used per lane except for the *fri;AtPRMT5* (Col) samples, in which 20 μg of total RNA was used (Fig. 2*D*). Samples were separated on a denaturing gel and transferred by capillary blotting to Nytron N membranes (Sigma–Aldrich). RNA was fixed to the membrane by using a Stratalinker UV cross-linker (Stratagene). Hybridization using a ^{32}P-labeled *AtPRMT5* probe corresponding to the first 300 bp of the coding sequence was performed in the QuickHyb solution from Stratagene according to their protocol. Membranes were exposed to film for 3 days at −80°C (Fuji Film Super Rx).

RT-PCR. First-strand cDNA synthesis was performed on 5 μg of RNA with the MLV-RT First-Strand Synthesis System (Promega) for RT-PCR and a primer containing the M13 primer sequence with an oligo dT extension (5′-GTAAAACGACGGCCAGTCCCT$_{15}$-3′). PCR amplification of the cDNA was performed with Ex-Taq (Takara). *FLM* (5′-CCTCCGGAAAACTCTATGACTCT-3′ and 5′-CGGTATTTTGTTGCCGGAGCTACTTC-3′), *MAF2* (5′-GGCTCCGGAAAACTCTA-CAAGTC-3′ and 5′-TGATGGTGATTACTTGAGCAGCGGA-3′), and *UBQ* (5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′ and 5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3′) were amplified by using the indicated primers and the following cycles: 95°C for 4 min followed by 22 cycles for *UBQ*, 38 cycles for *FLM*, and 34 cycles for *MAF2* of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s. Amplified fragments were separated on a 1.2% agarose gel.

Western Blot Analysis. Nuclear protein extracts were isolated from a chromatin preparation as described in the ChIP protocol in ref. 29. Proteins were separated on a 12.5% SDS/PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences). Total H4R3 symmetric dimethylation was determined by using anti-H4R3 symmetric dimethyl antibodies (Abcam, catalog no. 5823-200) using the recommended 1:2,000 dilution.

ChIP Assays. Chromatin samples were prepared as described (31). Immunoprecipitations were performed by using a ChIP assay kit (Millipore, catalog no. 17-295) following the manufacturer's suggested protocol modified as previously described (42). Antibodies against trimethyl H3K9 and trimethyl H3K27 were obtained from Upstate Biotechnology. Primers used to detect Ta3 and the FLC-P region were previously described (10). Primers used to detect NDPK2 chromatin are as follows: region A forward, 5′-TGGTATTAAAGCAGCAAATTGTGCGA-3′; region A reverse, 5′-AAGAACAAAGTTAATTGACATAAATA-3′; region B forward, 5′-ATGGTGGGAGCGACTGTAGTTAGTAA-3′; region B reverse, 5′-CAAACCATAGAAGCTACAAGGTGAGGAA-3′; region C forward, 5′-GAGCATTATAAGGATCTTAGTGCTA-3′; region C reverse, 5′-CCAACACCTTCCCAAGCCTT-

TACCA-3′. All ChIP assays were performed at least three times from at least two chromatin samples prepared from biological replicates.

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