

ARABIDOPSIS TRITHORAX1 Dynamically Regulates FLOWERING LOCUS C Activation via Histone 3 Lysine 4 Trimethylation ^W

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Trithorax function is essential for epigenetic maintenance of gene expression in animals, but little is known about *trithorax* homologs in plants. *ARABIDOPSIS TRITHORAX1* (*ATX1*) was shown to be required for the expression of homeotic genes involved in flower organogenesis. Here, we report a novel function of *ATX1*, namely, the epigenetic regulation of the floral repressor *FLOWERING LOCUS C* (*FLC*). Downregulation of *FLC* accelerates the transition from vegetative to reproductive development in *Arabidopsis thaliana*. In the *atx1* mutant, *FLC* levels are reduced and the *FLC* chromatin is depleted of trimethylated, but not dimethylated, histone 3 lysine 4, suggesting a specific trimethylation function of *ATX1*. In addition, we found that *ATX1* directly binds the active *FLC* locus before flowering and that this interaction is released upon the transition to flowering. This dynamic process stands in contrast with the stable maintenance of homeotic gene expression mediated by *trithorax* group proteins in animals but resembles the dynamics of plant *Polycomb* group function.

INTRODUCTION

Epigenetic mechanisms play crucial roles in shaping and maintaining cell identity and in patterning the body plan during development. Epigenetic information is partly carried by histone proteins in the form of reversible covalent modifications at their N-terminal tails. In *Drosophila melanogaster*, the *Polycomb* group (PcG) and *trithorax* group (trxG) proteins form higher-order complexes, which antagonistically repress and maintain the expression of homeotic genes (*HOX* genes), respectively (Simon and Tamkun, 2002). PcG and trxG complexes contain SET (for Suppressor of variegation 3-9, Enhancer of zeste, TRX) domain proteins that have histone methyltransferase (HMT) activity. They posttranslationally modify lysines on histones H3 and H4 (Lachner et al., 2004), thereby regulating the accessibility of the transcription machinery to the *HOX* gene clusters. These Lys methylation states have been classified as repressive and activating marks, depending on their effect on gene expression.

In recent years, several *Arabidopsis thaliana* PcG complexes were shown to repress their target genes via deposition of H3K27me3 marks (reviewed in Pien and Grossniklaus, 2007).

This supports a conservation of the PcG function between plants and animals. Consequently, if *trithorax* functions were also conserved during evolution, trxG proteins may antagonistically regulate PcG target genes. Consistently, two *Arabidopsis* PcG target genes, the flowering time regulator *FLOWERING LOCUS C* (*FLC*) and the floral homeotic gene *AGAMOUS* (*AG*), show an enrichment of H3K4me2 and H3K4me3 marks at their chromatin, which correlates with active transcription (Bastow et al., 2004; He et al., 2004; Schubert et al., 2006). In *Drosophila*, such marks are deposited by the trxG protein Trithorax (TRX) and in mouse by the mixed-lineage leukemia (MLL) protein. The presence of H3K4me marks suggests that *trithorax* homologs and their associated functions exist in *Arabidopsis*.

Five close homologs of TRX and MLL were identified in the *Arabidopsis* genome and named *ARABIDOPSIS TRITHORAX* (*ATX1-5*) (Alvarez-Venegas and Avramova, 2001; Baumbusch et al., 2001). *ATX1* is predicted to contain a SET domain (Alvarez-Venegas et al., 2003) that has both histone binding and HMT activity (Rea et al., 2000; Katsani et al., 2001). In vitro assays demonstrated that H3K4 is a substrate for *ATX1*'s HMT activity, while mutant isoforms of *ATX1* lacking part of the SET domain have no activity (Alvarez-Venegas et al., 2003). Loss of *ATX1* leads to flower homeotic defects and affects leaf morphogenesis (Alvarez-Venegas et al., 2003). Recently, *ATX1* was shown to bind *AG* chromatin and to be required for H3K4me3 deposition at this locus (Saleh et al., 2007).

Transcriptional profiling in the *atx1* mutant allowed us to identify *FLC*, a flowering time regulator, as a putative *ATX1*

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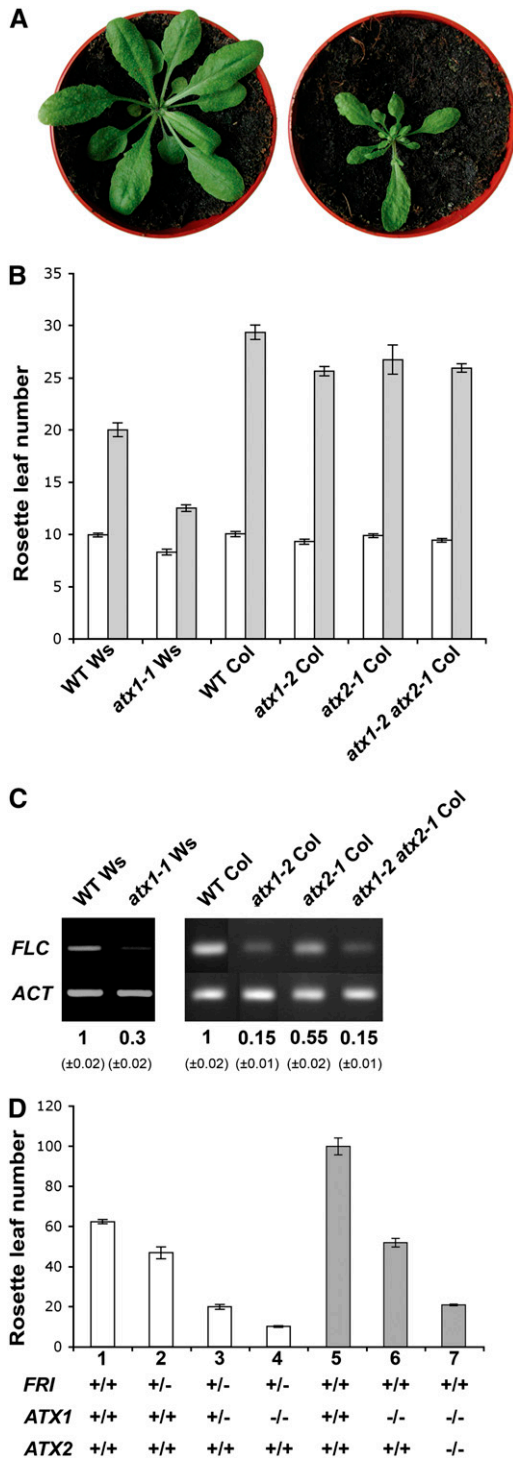


Figure 1. Characterization of *atx* Mutants.

(A) At flowering, the *atx1* (Ws) rosette leaf ([A], right) is smaller than the wild type ([A], left), with a reduction in the leaf number in *atx1* compared with the wild type.

(B) The average number of rosette leaves at flowering, a measure of flowering time, is reduced in *atx1* and *atx2* mutants. Open bars, leaf number at flowering under long-day conditions; gray bars, short-day

target gene. *FLC* encodes a MADS domain transcription factor that functions as a repressor of the floral transition. Transcriptional regulation of *FLC* has been well studied and shown to be associated with chromatin modifications, but little was known about the activation and maintenance of expression of this central gene. Therefore, we studied *FLC* regulation as a model to decipher the molecular mechanism of *trithorax* function in plants and to gain insight into novel functions of the *ATX1* gene. We showed that both single and double mutation of *ATX1* and its closest homolog *ATX2* (Alvarez-Venegas and Avramova, 2001) lead to early flowering, correlating with a reduction of *FLC* transcripts levels. *ATX1* and its target gene *FLC* are coexpressed in a spatio-temporal manner. Using chromatin immunoprecipitation (ChIP), we showed that *ATX1* binds at the *FLC* locus and its presence correlates with H3K4me3 modifications. Furthermore, ChIP analyses revealed that *ATX1* not only activates *FLC* but it also prevents its repression, since H3K27me2 repressive marks are deposited in the absence of *ATX1* function. Finally, our study identified *ATX1* as a direct transcriptional activator of *FLC*.

RESULTS

atx1 and *atx2* Are Early-Flowering Mutants Affecting the *FLC* Expression Level

The *atx1-1* mutation was previously reported to delay the transition to flowering (Alvarez-Venegas et al., 2003). These studies, however, did not quantify the changes in flowering time or leaf number at bolting. Therefore, we investigated the *atx1-1* mutant in more detail to decipher how the flowering transition was affected. In contrast with previously published work (Alvarez-Venegas et al., 2003), under our growth conditions *atx1* mutations

conditions. All data are presented as means \pm SE ($n = 15$ to 20 ; $P < 0.05$ using Student's *t* test).

(C) RT-PCR quantification of *FLC* transcripts in the wild type and in *atx1* and *atx2* mutants. Left panel, wild type (Ws) and *atx1-1* (Ws) mutant; right panel, wild type (Col), *atx1-2* (Col), and *atx2-1* (Col) mutants. Numbers (\pm SE) refer to *FLC* transcript level relative to the wild type of three independent biological replicate experiments. *ACT*, actin loading control.

(D) Average number of rosette leaves at flowering. *atx1-1* (Ws) mutants were crossed with Col plants (Col^{Sf-2}), into which the wild-type San Feliu-2 (*Sf-2*) flowering-time locus *FRIGIDA* (*FRI^{Sf-2}*) had been introgressed (Lee and Amasino, 1995) (white columns). *atx1-2 atx2-1* (Col) double mutants were crossed with Col^{Sf-2} (gray columns). Columns 1 to 4 represent segregating F2 populations; the origin of the relevant alleles is indicated. Columns 5 to 7 are from a homogeneous background resulting from crosses between Col^{Sf-2} and Col. 1, *FRI^{Sf-2}* (Col^{Sf-2}) *ATX1* (Col^{Sf-2}) ($n = 12$); 2, *FRI^{Sf-2}/fri* (Col^{Sf-2} /Ws) *ATX1* (Col^{Sf-2}) ($n = 46$); 3, *FRI^{Sf-2}/fri* (Col^{Sf-2} /Ws) *ATX1/atx1* (Col^{Sf-2} /Ws) ($n = 67$); 4, *FRI^{Sf-2}/fri* (Col^{Sf-2} /Ws) *atx1-1* (Ws) ($n = 38$); 5, *FRI^{Sf-2}* (Col^{Sf-2}) *ATX1* (Col) *ATX2* (Col); 6, *FRI^{Sf-2}* (Col^{Sf-2}) *atx1-2* (Col) *ATX2* (Col); 7, *FRI^{Sf-2}* (Col^{Sf-2}) *atx1-2* (Col) *atx2-1* (Col). In a heterozygous *FRI^{Sf-2}/fri* background, the introduction of one *atx1* copy results in a reduced number of leaves at flowering time. The suppression of *FRI* had a greater effect on flowering time in the *atx1* (Ws) background. In a Col^{Sf-2} background carrying the *FRI^{Sf-2}* allele, the *atx1-1 atx2-1* double mutant suppressed the late-flowering phenotype more dramatically than the *atx1-2* single mutant. All data are presented as means \pm SE ($n = 12$ to 67 ; $P < 0.05$ using Student's *t* test).

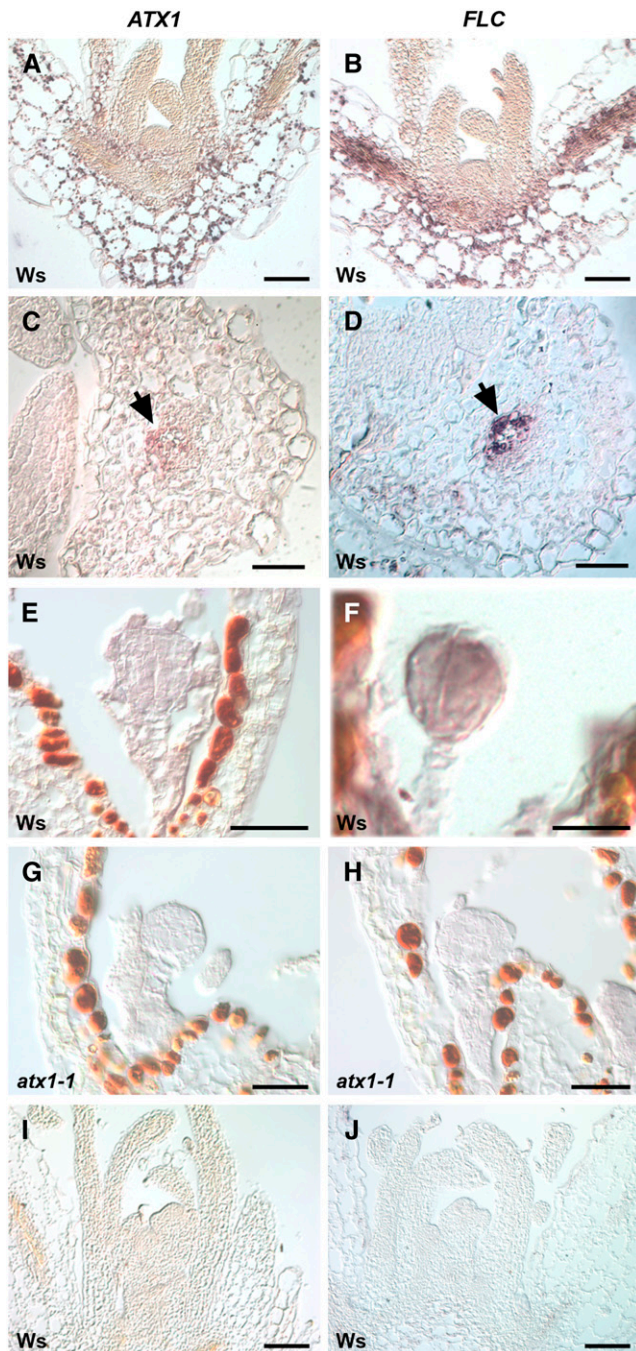


Figure 2. Spatio-Temporal Expression Patterns of *ATX1* and *FLC* in Wild-Type and *atx1-1* Tissue as Assayed by in Situ Hybridization.

(A), (C), (E), (G), and (I) Sections probed with an antisense *ATX1* probe. (B), (D), (F), (H), and (J) Sections probed with an antisense *FLC* probe. Wild-type tissue sections hybridized with sense probes for *ATX1* and *FLC* gave no signal at any developmental stage.

(A) and (B) Ten-day-old seedlings with *ATX1* and *FLC* transcripts accumulating in the vasculature and the hypocotyl.

(C) and (D) Ten-day-old seedlings. Cross sections of the first pair of leaves, with *ATX1* and *FLC* transcripts accumulating in the vasculature (arrows).

led to an early-flowering phenotype in the rapid-flowering accessions Wassilewskija (*Ws*) and Columbia (*Col*), as measured by the number of rosette leaves at bolting (Figures 1A and 1B). *atx1-1* mutants flowered early under both long-day and short-day conditions, showing that *ATX1* is involved in repressing the transition to flowering, independently of the photoperiod.

To investigate the molecular basis of the early-flowering phenotype of *atx1-1* mutant plants, we performed RNA profiling experiments with 10-d-old *atx1-1* and wild-type seedlings using the Affymetrix ATH1 GeneChip. To reveal whether *trxG* and *PcG* proteins play similar antagonistic roles as they do in animals (Simon and Tamkun, 2002), we looked for differentially expressed genes that had already been classified as *PcG* target genes. With these criteria, we found that the floral regulator *FLC*, a *PcG* target gene, had greatly reduced steady state transcript levels in the *atx1-1* mutant (3.4-fold decrease; see Supplemental Table 1 and Supplemental Table 2 online). This reduction was confirmed by RT-PCR in both *atx1-1* and *atx1-2* (*Col*) homozygous mutants (Figure 1C).

We further investigated the impact of *atx1* mutations on flowering time by crossing *atx1-1* and *atx1-2* mutants with a line containing an active *FRIGIDA* (*FRI*) allele (Lee and Amasino, 1995). The presence of the active *FRI* allele is associated with *Arabidopsis* late-flowering accessions and results in a high level of *FLC* expression. Loss of *ATX1* strongly suppressed the late-flowering effect of *FRI* in a dosage-dependent manner (Figure 1D). Thus, *ATX1* is required for the increased expression of *FLC* that results from overexpression of *FRI* in the line containing an active *FRI* allele.

Mutation of the closest homolog of *ATX1*, *ATX2* (Alvarez-Venegas and Avramova, 2001), revealed a role for *ATX2* in *FLC* regulation (Figure 1C). In a *FRI* background, the *atx1-2 atx2-1* double mutant suppressed the late-flowering phenotype more dramatically than in the *atx1-2* single mutant (Figure 1D), suggesting that *ATX1* and *ATX2* play a partially redundant role in activating *FLC*.

***ATX1* and *FLC* Are Spatio-Temporally Coexpressed**

Since we found that *ATX1* and *ATX2* regulate *FLC*, we analyzed the spatio-temporal expression of these three genes by in situ hybridization. If *ATX1* and *ATX2* directly regulate *FLC* during the plant life cycle, then the spatio-temporal expression patterns of *ATX1*, *ATX2*, and *FLC* are expected to overlap. In situ hybridization analyses for *ATX1* and *FLC* in 10-d-old seedlings revealed expression of both genes in the vasculature of the cotyledons, hypocotyls, and the first pair of leaves (Figures 2A to 2D). The pattern of *FLC* mRNA accumulation reproduces the expression pattern of the reporter gene *uidA*, encoding β -glucuronidase (*GUS*), translationally fused to *FLC* (*FLC-GUS*) (Bastow et al., 2004). Both *ATX1* and *FLC* transcripts were present in overlapping

(E) and (F) Wild-type globular embryos showing expression of both *ATX1* and *FLC*.

(G) and (H) In *atx1-1* globular embryos, neither *ATX1* nor *FLC* transcripts are detectable.

(I) and (J) At flowering, neither *ATX1* nor *FLC* message is detectable in the wild-type vasculature.

patterns during embryogenesis (Figures 2E and 2F). *FLC* transcript was not detected in *atx1-1* embryos, suggesting that *ATX1* is necessary to activate *FLC* well before the floral induction pathways are active (Figures 2G and 2H). Just prior to flowering, a strong reduction of both *ATX1* and *FLC* transcript levels occurred in the vasculature of wild-type plants (Figures 2I and 2J). The overlap of the spatio-temporal expression patterns of the two genes is consistent with a direct regulation of *FLC* by *ATX1*. These data were confirmed by a cross between a *FRI* line containing an *FLC-LUCIFERASE (FLC-LUC)* translational fusion (Myline et al., 2004) and the *atx1-2* mutation (Figures 3A and 3B). In *FRI FLC-LUC* plants, *FLC* was highly expressed, as indicated by the *FLC-LUC* signal in the vasculature and the shoot apex (Figure 3A). In *FRI FLC-LUC atx1-2* lines, *FLC* expression was strongly reduced in the vasculature (Figure 3B). Interestingly, *ATX1* expression could not be detected in the shoot apical meristem of wild-type plants (Figure 2A), and mutations in *ATX1* did not lead to a loss of *FLC* expression in that tissue (Figure 3B), suggesting that *FLC* expression in the shoot apical meristem is positively regulated by some other factor(s). We investigated the expression pattern of *ATX2* at the same developmental stage. *ATX2* was expressed in the vasculature and, unlike *ATX1*, was also detected in the shoot apical meristem (see Supplemental Figure 1 online). In *FRI FLC-LUC atx1-2 atx2-1* lines, *FLC* expression was strongly reduced in the vasculature and in the shoot apex (Figure 3C). However, *FLC* expression was still detectable in the shoot apical meristem of *FRI FLC-LUC atx1-2 atx2-1* lines, confirming that *ATX1* and *ATX2* play their major role in the vasculature.

ATX1 Is Required for the Deposition of H3K4me3 Marks at the *FLC* Locus

To address whether *ATX1*-dependent histone modifications are involved in the regulation of *FLC*, we analyzed chromatin modifications at the *FLC* locus by ChIP at three regions surrounding the translational start codon (Figure 4A). These regions are essential for *FLC* transcription and function (Bastow et al., 2004; Kim et al., 2005), and H3K4me3 marks in these regions correlate with *FLC* transcription (He et al., 2004). We found that

H3K4me3 levels were reduced in region B and undetectable in region A in *atx1-1* mutants (Figure 4B) compared with wild-type plants at the same developmental stage. Just prior to floral induction, H3K4me3 levels decreased in wild-type plants to levels similar to those in *atx1-1* mutant seedlings. Taken together, these findings suggest that *ATX1* is required for the establishment of the H3K4me3 mark at the *FLC* locus to promote and/or maintain a transcriptionally active state.

In plants and animals, H3K4 can be either, mono-, di-, or trimethylated. These three epigenetic marks can be interpreted differently by the transcription machinery depending on the organism (Fuchs et al., 2006). Therefore, we quantified in parallel H3K4me2 and H3K4me3 marks at the *FLC* locus in wild-type and *atx1-1* plants. Surprisingly, region B, which covers the transcription start site of *FLC*, displayed elevated levels of H3K4me2 in 10-d-old *atx1-1* seedlings compared with the wild type (Figure 4B). In regions A and C, this mark was almost at the same level as in wild-type plants. At later developmental stages, prior to the flowering transition, a similar pattern could be observed. Altogether, the loss of *ATX1* activity strongly impaired the deposition of H3K4me3 marks but did not suppress the deposition of H3K4me2 marks at *FLC* chromatin. These findings suggest that the function of *ATX1* seems to be specific for the deposition of the H3K4me3 mark.

Loss of H3K4me3 marks at the *FLC* locus induces a gain of H3K27me2 marks

Since in the *atx1-1* mutant, *FLC* is depleted in the H3K4me3 activation mark and is transcriptionally repressed, we measured the levels of H3K27me2 and H3K27me3, two marks previously shown to correlate with *FLC* repression (Bastow et al., 2004; Sung et al., 2006). In the regions A and B, an increased level of H3K27me2 was observed in *atx1-1* mutant seedlings compared with wild-type seedlings at the same stage. H3K27me2 levels also increased, although less dramatically, prior to flowering. In wild-type plants, the level of H3K4me3 inversely correlated with the level of H3K27me2. However, H3K27me2 marks were still present on *FLC* chromatin in regions A and B during active

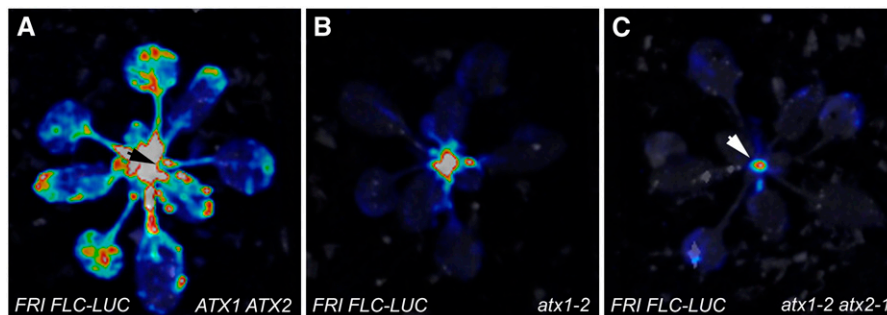


Figure 3. *FLC-LUC* Expression Pattern in *FRI*, *FRI atx1-2*, and *FRI atx1-2 atx2-1* Plants.

(A) In *FRI FLC-LUC* lines, *FLC* is highly expressed in the vasculature and the shoot apex (arrow).

(B) In *FRI FLC-LUC atx1-2* lines, *FLC* expression is strongly reduced in the vasculature.

(C) In *FRI FLC-LUC atx1-2 atx2-1* lines, *FLC* expression is strongly reduced in the vasculature and in the shoot apex (arrow).

Twenty-five-day-old plants were analyzed for *FLC-LUC* expression for all lines analyzed. Two biological replicates were performed, growing side by side 10 plants of each genotype.

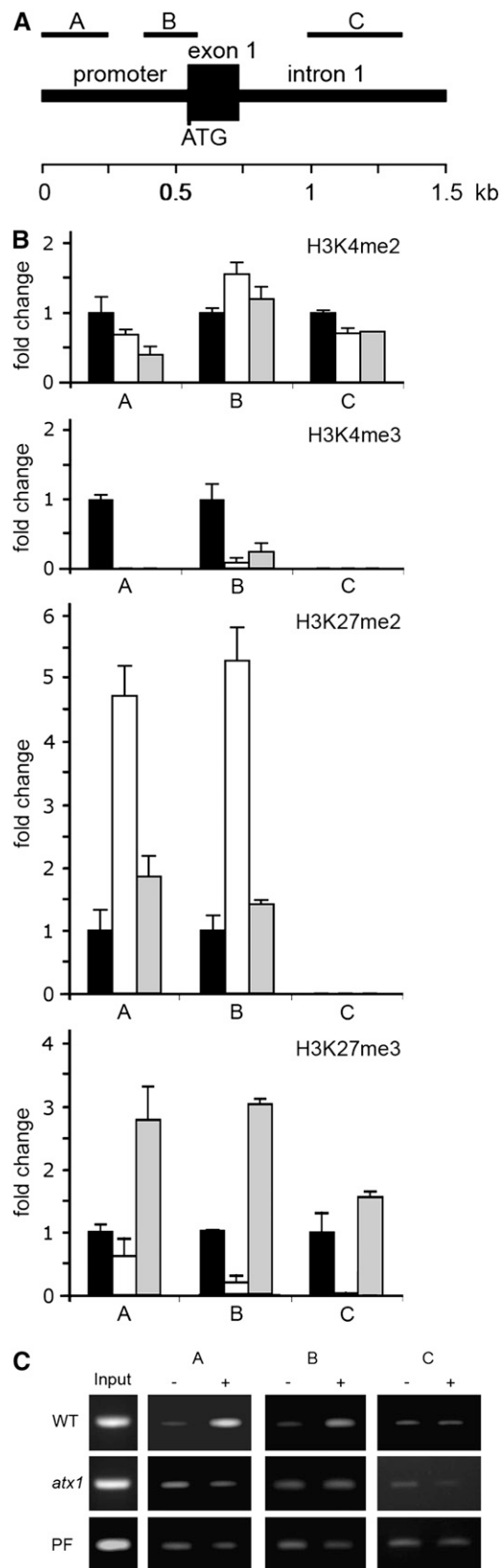


Figure 4. Histone Modifications and ATX1 Binding at the *FLC* Locus.

transcription of the gene prior to the flowering transition. Similarly, low H3K27me3 levels could be detected in wild-type seedlings at the *FLC* chromatin in all regions investigated (Figure 4B). It is worth noting that the presence of this repressive mark together with the activating mark H3K4me3 does not prevent active transcription of *FLC*. The level of the H3K27me3 mark, however, substantially increased in plants prior to flowering, which correlates with *FLC* repression at that developmental stage. This is consistent with the observation that H3K27me3 deposition by plant PcG proteins correlates with transcriptional repression of the *Arabidopsis* PcG target genes *MEDEA* (*MEA*), *PHERES1* (*PHE1*), *AG*, *SHOOTMERISTEMLESS* (*STM*), and *AGAMOUS-LIKE 19* (*AGL19*) (Gehring et al., 2006; Jullien et al., 2006; Makarevich et al., 2006; Schönrock et al., 2006; Schubert et al., 2006).

ATX1 Directly Interacts with *FLC* Chromatin to Regulate Its Transcription

The ability of *ATX1* to promote H3K4me3 deposition suggests that *ATX1* directly interacts with the *FLC* locus to modify its chromatin state via its HMT activity (Alvarez-Venegas et al., 2003). We investigated this possibility by ChIP (Figure 4C) using an antibody raised against *ATX1*. We found that *ATX1* was enriched at the *FLC* chromatin (regions A and B) in wild-type seedlings relative to *atx1-1* mutants. In the wild type, *ATX1* was not enriched in region C, which is consistent with the absence of the H3K4me3 mark in that region (Figure 4B). These data show that *ATX1* binding to the *FLC* chromatin correlates with the deposition of H3K4me3 marks. In wild-type plants at the floral transition, when *FLC* is downregulated, *ATX1* binding at the *FLC* locus could not be detected. This observation suggests that *ATX1* dynamically binds the *FLC* locus to regulate its transcription.

DISCUSSION

The *trxG* Genes *ATX1* and *ATX2* Are Required to Activate *FLC* Expression

Our study provides strong evidence that *ATX1*, a homolog of the *Drosophila* *trx* protein, is required to control flowering transition and acts to upregulate *FLC* expression. *ATX1* acts downstream of, or in parallel with, *FRI* in an interdependent manner. It also

(A) Genomic structure of the *FLC* promoter and regions investigated by ChIP. The thick lines represent the 5' untranslated region and intron 1, while the black box represents the translated region of exon 1. Regions amplified by PCR are labeled A to C.

(B) Relative levels of histone modifications in *FLC* chromatin were analyzed by PCR from at least three replicate ChIP assays using H3K4me2-, H3K4me3-, H3K27me2-, and H3K27me3-specific antibodies. Black bars, 10-d-old *Ws* seedlings; open bars, 10-d-old *atx1-1* mutant seedlings; gray bars, 16-d-old wild-type plants prior to flowering. Means are calculated based on at least three independent experiments and are given with bars indicating 1 SE.

(C) ChIP assay using an *ATX1*-specific antibody. Regions A, B, and C were examined for *ATX1* enrichment in *FLC* chromatin. +, *ATX1* antibody; -, no antibody controls. Regions A and B showed enrichment of *ATX1* in *Ws* seedlings. PF, 16-d-old *Ws* plants prior to flowering.

acts directly on *FLC* and binds to its promoter and transcription start site regions. Many regulators of *FLC* transcription have been described (reviewed in He and Amasino, 2005), but, unlike *ATX1*, these do not appear to modify *FLC* chromatin directly. The putative HMT *EARLY FLOWERING IN SHORT DAYS (EFS)*, a homolog of the *trxG Drosophila* protein Absent small homeotic disks1 (*ASH1*) (Tripoulas et al., 1994), was shown to be necessary for the deposition of H3K4me3 marks at *FLC* chromatin of winter annual accessions (He et al., 2004). In such accessions, vernalization (extended exposure to cold) is required to activate the *VRN Polycomb Repressive Complex 2 (VRN-PRC2)* (Levy et al., 2002; Kim et al., 2005), which represses *FLC*, leading to flowering. However, in the commonly studied rapid-flowering accession *Col*, vernalization is not required for flowering, and mutations in *EFS* do not affect the level of H3K4me3 at the *FLC* locus (He et al., 2004). In this context, our data provide evidence that *ATX1* regulates *FLC* transcription in the rapid-flowering accessions *Ws* and *Col*. Additionally, in lines containing an active *FRI* allele, which mimic winter annual accessions, the *atx1-1 atx2-1* double mutant suppressed the late-flowering phenotype, suggesting that genes of the *ATX* family are also central for *FLC*-mediated regulation in winter annual accessions. How *ATX1*, *ATX2*, and *EFS* collaborate in this process remains unclear and would require additional investigations. Our data suggest that *ATX1* and *ATX2* are involved in the same pathway since the early-flowering phenotype observed in single mutants is not

more severe in the *atx1-2 atx2-1* double mutant. However, in the *FRI* background, mutation of both genes leads to a shorter vegetative phase compared with *atx1* single mutants in the same background. This may be explained by delayed transcription of *ATX2*, whose expression is detected later than *ATX1* during the vegetative phase and which may have a stronger impact in winter annual accessions than in rapid-flowering accessions.

PcG and *trxG* Proteins Dynamically Regulate *FLC* Expression

In contrast with animals, where PcG and *trxG* proteins play a role in the permanent repression or activation of genes whose expression state was determined by other factors, in plants, PcG and *trxG* proteins dynamically interact in the regulation of target genes, such as *FLC*, during the plant life cycle (reviewed in Pien and Grossniklaus, 2007). In wild-type plants at the floral transition, when *FLC* transcripts are no longer detectable in the apex and neighboring vasculature, *ATX1* binding at the *FLC* locus could not be detected (Figure 4C). This indicates that *ATX1* dynamically binds the *FLC* locus to regulate its transcription. In *Drosophila*, *TRX* together with members of the *PRC1/2* complexes are constitutively bound to the *HOX Ultrathorax (Ubx)* locus independent of whether the *Ubx* gene is actively transcribed or not (Papp and Müller, 2006). In contrast with the situation in *Drosophila* and mammals, *ATX1* binding is not stable,

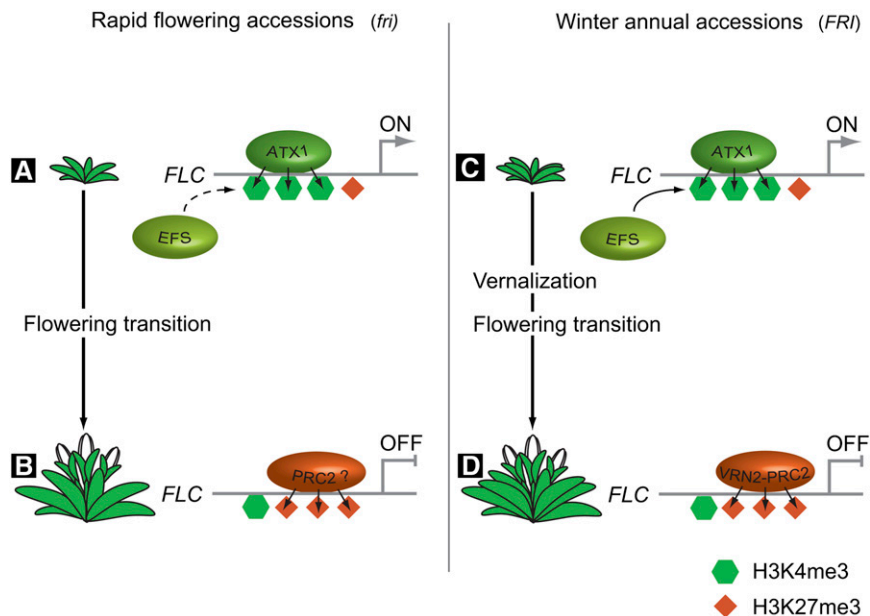


Figure 5. Model for Dosage-Dependent Regulation of *FLC* Expression by Chromatin Modifications.

(A) In rapid-flowering accessions (*fri* background), *FLC* is activated by *ATX1* via the deposition of H3K4me3 marks at the *FLC* 5' untranslated region during the vegetative phase.

(B) The H3K27me3 repressive mark is present but does not prevent *FLC* expression. *EFS* is required to prevent early flowering but does not modify the level of H3K4me3 marks at the *FLC* locus (Kim et al., 2005). The removal of H3K4me3, together with an increased level of H3K27me3 mark deposited by a still unknown PRC2 complex, leads to *FLC* repression and subsequent flowering.

(C) In winter annual accessions (*FRI* background), *ATX1* together with *EFS* activates *FLC* expression via the deposition of H3K4me3 marks.

(D) A prolonged cold treatment (vernalization) induces the *VRN2-PRC2* complex, which in turn represses *FLC* via the deposition of H3K27me3 marks.

which points to a dynamic function of trxG complexes in plants. This dynamic process is reflected by the removal of previously deposited H3K4me3 at the *FLC* promoter during the transition from vegetative to reproductive development (Figure 4B). Recently, dynamic regulation was also demonstrated for PcG proteins (Baroux et al., 2006). Together, these data suggest that plant PcG and trxG proteins affect a wide range of gene expression programs and potentially contribute to plant developmental plasticity.

***FLC* Is Regulated through Dosage-Dependent Interactions of Activating and Repressive Histone Modifications**

The results presented here highlight the importance of the H3K4me3 modification mediated by ATX1 for the transcriptional activation of *FLC*: the levels of H3K4me2 in the *atx1* mutants are clearly not sufficient in this context. However, we cannot rule out that the deposition of H3K4me2 marks does not play a role in active *FLC* transcription. A recent study provided evidence on the requirement of *FCA* together with *FLOWERING LOCUS D (FLD)* to mediate H3K4 demethylation of *FLC* in its central region and, thus, to silence the gene (Liu et al., 2007). By contrast, at several *Arabidopsis* loci, the H3K4me2 mark was shown to be associated with the H3K27me2 mark, independent of whether the associated genes were actively transcribed or not (Alvarez-Venegas and Avramova, 2005).

Surprisingly, our data showed that repressive H3K27me2 and H3K27me3 modifications were present at the *FLC* locus during active transcription and *FLC* silencing. This observation is in agreement with the whole-genome analysis of H3K27me3 distribution in the *Arabidopsis* Ws accession, where this mark was detected at *FLC* chromatin during active transcription (Zhang et al., 2007). However, it is notable that the levels of H3K27me2 and H3K27me3 marks at *FLC* are always lower than the levels observed in plants prior to flowering, where *FLC* is silenced (Figure 4B). The presence of repressive marks at the *FLC* locus during its active expression can be interpreted as basal levels that are not sufficient to repress *FLC* expression in that context. Therefore, our results suggest a mechanism where the active or repressive state of *FLC* expression depends on the accumulation of repressive and activating marks in a dosage-dependent manner (i.e., the expression of *FLC* correlates with the deposition of H3K4me3 marks and basal levels of H3K27me2 and H3K27me3 marks at the *FLC* promoter). Conversely, repression of *FLC* is associated with the removal of H3K4me3 marks and a substantial increase of H3K27me3 marks at the *FLC* promoter (see model in Figure 5).

The absence of H3K4me3 marks at *FLC* chromatin in the *atx1-1* mutant is correlated with an accumulation of H3K27me2 (Figure 4B), a mark associated with *FLC* gene repression (Bastow et al., 2004). This suggests the presence of a default mechanism that represses *FLC* transcription in the absence of H3K4me3 marks. A similar mechanism was described in *Drosophila*, where the trxG proteins ASH1 and TRX have been proposed to counteract PcG repression, either by histone binding and/or H3K4-trimethylation, which subsequently prevents the binding of PcG proteins to *HOX* genes (Klymenko and Müller, 2004). The simultaneous binding of TRX and PcG proteins at the *Ubx* locus challenged this hypothesis (Papp and Müller, 2006). Recently, ASH1 binding at the *Ubx* locus

was shown to correlate with H3K4me3 deposition and to occur only when *Ubx* is transcribed (Papp and Müller, 2006). Therefore, ASH1 was proposed to counteract PcG repression via the deposition of the H3K4me3 marks, which subsequently restricts H3K27 methylation in the promoter and coding regions. Whether or not this mechanism is conserved in plants will require more investigations; however, our study provides evidence for a similar mechanism in *Arabidopsis* using different histone marks.

In the *atx1-1* mutant background, the accumulation of H3K27me3 marks was reduced in the promoter and the first intron, arguing for the requirement of ATX1 and/or the presence of H3K4me3 marks for the deposition of this repressive mark. A similar result was recently observed at the *AG* locus, where ATX1 is required for the trimethylation of H3K27 in the promoter and the downstream coding region (Saleh et al., 2007). By contrast, the *atx1-1* mutation results in an increased level of another repressive mark at *FLC*, H3K27me2, showing that ATX1 activity is not required for the deposition of this repressive mark. This suggests that in the absence of the H3K4me3 mark in this region, the H3K27me3 mark is not required to repress *FLC*.

In summary, we demonstrate that ATX1 directly regulates the floral regulator *FLC* by mediating the H3K4me3 modification. Additionally, we show that H3K4me3 deposition is accompanied by a decrease in H3K27me2 levels at the *FLC* locus. Thus, we propose that the developmentally regulated binding of ATX1 and trimethylation of H3K4 at *FLC* chromatin counteract *FLC* silencing. Our study also shows that transition to flowering correlates with the release of ATX1 from the *FLC* locus and an increase of the level of H3K27me3 repressive marks, of which a critical level is required to achieve full repression of *FLC* (Shindo et al., 2006). This time- and dosage-dependent regulation resembles the vernalization process, where prolonged exposure to cold leads to progressive silencing of *FLC* (Chouard, 1960; Lang, 1965). Chromatin-mediated regulation of *FLC*, and probably other genes, is not an all-or-nothing process and fine-tuning may be achieved through different levels of histone modifications.

METHODS

Plant Material and Growth Conditions

Seeds, wild-type Ws, and *atx1-1* (Ws) (Alvarez-Venegas et al., 2003), wild-type Col, *atx1-2* (Col) (SALK_149002), *atx2-1* (Col) (SALK_074806), and *FRI* (Col^{Sf-2}) plants, in which the flowering-time locus *FRI* has been introgressed from the Sf-2 accession into a Col background (Lee and Amasino, 1995), were grown on Murashige and Skoog media with 15 g/L of sucrose at 4°C for 2 d under short-day conditions (8/16 h day/night) with 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ white light and then transferred to 20°C under either long-day (16/8 h day/night) or short-day conditions with 57 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ white light. Luciferase imaging was as described by Mylne et al. (2004), and the images were obtained using a NightOwl imaging system (Berthold Technologies).

atx1-1 plants were genotyped using SP26 (forward) 5'-TCTATG-CAGCTCTTTGCTAATTGG-3' and TDNA-LB SP11 (reverse) 5'-GAT-GCACTCGAAATCAGCCAATTTTAGAC-3' or SP26 (forward) and SP27 (reverse) 5'-AGCCCAGAGCATGAGCTTACC-3' for the wild-type *ATX1* gene. *atx1-2* plants were genotyped using JM341 (forward) 5'-GGTA-TAGCTCATGCTCTGGGC-3' and SALK-LB (reverse) 5'-CCAACTGGA-ACAACACTCAAC-3' or JM341 (forward) and JM340 (reverse) 5'-TCT-CTTTTGTGGACTTGCTGTG-3' for the wild-type *ATX1* gene. *atx2-1*

plants were genotyped using JM345 (forward) 5'-GCTGCAAAGAA-CAAACCTCTCC-3' and SALK-LB (reverse) or JM345 (forward) and JM346 (reverse) 5'-AGGCCACCAATAGCTGACAAG-3' for the wild-type *ATX2* gene.

RT-PCR Analyses

RT-PCR quantifications were performed with 10-d-old seedlings. RNA was isolated with the Trizol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR for *FLC* was performed using *FLC*-specific primers SP135 (forward) 5'-TTGGATCAGTCAAAAGC-3' and SP136 (reverse) 5'-AGTAGTGGGAGAGTACACGGG-3', and *ACTIN2* (*ACT2*) control primers were SP105 (forward) 5'-GCCCTCGTTTGTGGGAATGG-3' and SP106 (reverse) 5'-AAGCCTTGGATCTTGAGAGC-3'. Signal intensities using ethidium bromide staining (0.4 µg/mL) were normalized relative to *ACTIN2* PCR products with ImageQuant software (Molecular Dynamics). These results are representative of three independent biological replicate experiments. Fold changes are the mean of three independent quantifications from three independent RNA extractions. Primer efficiency was tested to quantify *FLC* and *ACT2* PCR product in the logarithmic phase.

In Situ Hybridization

Fixation and hybridization were performed as previously described (Köhler et al., 2003). Primers used to make the probes were as follows: *ATX1*, SPG63 (forward) 5'-AGCTGGATCCAGTCTGATGTCTAAGAAGG-3' and SPG64 (reverse) 5'-ACGTGAATTCCTTACACCTTCTTAAACC-3'; *ATX2*, SPG54 (forward) 5'-ATGCGGATCCGGAAGATCAGTCCCTGATAC-3' and SPG55 (reverse) 5'-AGCTGAATTCCTTCTGAAGTTGATCCATC-3'; *FLC*, SPB1 (forward) 5'-AGCTGGATCCTTGGATCATCAGTCAAAAGC-3' and SPB2 (reverse) 5'-AGCTGAATTCAGTAGTGG GAGAGTACCCGG-3'.

ChIP

ChIP was performed on 10-d-old seedlings and 16-d-old plants prior to flowering, grown under long-day conditions, as previously described (Köhler et al., 2003). Antibodies used were H3K4me2 (Upstate), H3K4me3 (Upstate), H3K27me2 (Upstate), H3K27me3 (Upstate), and ATX1 (GenScript). Primers for *ACTIN2/7* and for *FLC* regions A (Bastow et al., 2004), B (He et al., 2004), and C (Bastow et al., 2004) were as previously described. PCR conditions were similar to the ones used by Bastow et al. (2004) and He et al. (2004), where analysis to show the amplification efficiency of all primer pairs used in the chromatin immunoprecipitation analysis has been published. Signal intensities using ethidium bromide staining (0.4 µg/mL) were normalized relative to *ACTIN 2/7* PCR products with ImageQuant software (Molecular Dynamics), and the fold changes are expressed relative to the value of wild-type seedlings. Means are given with bars indicating 1 SE.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: AG, At4g18960; AGL19, At4g22950; ATX1, At2g31650; ATX2, At1g05830; EFS, At1g77300; FCA, At4g16280; FLD, At3g10390; FLC, At5g10140; FRI, At4g00650; MEA, At1g02580; PHE1, At1g65330; STM, At1g62360.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Spatio-Temporal Expression Patterns of *ATX2* in Wild-Type 10-d-Old Seedlings Assayed by In Situ Hybridization.

Supplemental Table 1. Steady State Message Levels in the *atx1-1* Homozygous Mutant Compared with Wild-Type Ws.

Supplemental Table 2. Downregulated Genes in the *atx1-1* Homozygous Mutant Compared with Wild-Type Ws.

Supplemental References.

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ARABIDOPSIS TRITHORAX1 Dynamically Regulates *FLOWERING LOCUS C* Activation via Histone 3 Lysine 4 Trimethylation

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