

# ARABIDOPSIS TRITHORAX-RELATED3/SET DOMAIN GROUP2 is Required for the Winter-Annual Habit of *Arabidopsis thaliana*

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The winter-annual habit of *Arabidopsis thaliana* requires active alleles of *FLOWERING LOCUS C (FLC)*, which encodes a potent flowering repressor, and *FRIGIDA (FRI)*, an activator of *FLC*. *FLC* activation by *FRI* is accompanied by an increase in specific histone modifications, such as tri-methylation of histone H3 at lysine 4 (H3K4me3), and requires three H3K4 methyltransferases, the *Drosophila* Trithorax-class ARABIDOPSIS TRITHORAX1 (ATX1) and ATX2, and yeast Set1-class ATX-RELATED7/SET DOMAIN GROUP25 (ATXR7/SDG25). However, lesions in all of these genes failed to suppress the enhanced *FLC* expression caused by *FRI* completely, suggesting that another H3K4 methyltransferase may participate in the *FLC* activation. Here, we show that ATXR3/SDG2, which is a member of a novel class of H3K4 methyltransferases, also contributes to *FLC* activation. An ATXR3 lesion suppressed the enhanced *FLC* expression and delayed flowering caused by an active allele of *FRI* in non-vernalized plants. The decrease in *FLC* expression in *atxr3* mutants was accompanied by reduced H3K4me3 levels at *FLC* chromatin. We also found that the rapid flowering of *atxr3* was epistatic to that of *atxr7*, suggesting that ATXR3 functions in *FLC* activation in sequence with ATXR7. Our results indicate that the novel-class H3K4 methyltransferase, ATXR3, is a transcriptional activator that plays a role in the *FLC* activation and establishing the winter-annual habit. In addition, ATXR3 also contributes to the activation of other *FLC* clade members, such as *FLOWERING LOCUS M/MADS AFFECTING FLOWERING1 (FLM/MAF1)* and *MAF5*, at least partially explaining the ATXR3 function in delayed flowering caused by non-inductive photoperiods.

**Keywords:** ARABIDOPSIS TRITHORAX • Flowering • FLOWERING LOCUS C • Histone methylation • Winter-annual Arabidopsis.

**Abbreviations:** ABRC, Arabidopsis Biological Resource Center; ACT, ACTIN; AG, AGAMOUS; Ash1, Absent, small or homeotic discs 1; ATX, ARABIDOPSIS

TRITHORAX; ATXR, ATX-RELATED; ChIP, chromatin immunoprecipitation; CLF, CURLY LEAF; Col, Columbia; ELF7, EARLY FLOWERING7; EFS, EARLY FLOWERING IN SHORT DAYS; E(z), Enhancer of zeste; FLC, FLOWERING LOCUS C; FLM, FLOWERING LOCUS M; FRI, FRIGIDA; INRA, National Institute for Agricultural Research; KYP, KRYPTONITE; MAF, MADS AFFECTING FLOWERING; ML, maximum likelihood; NCBI, National Center for Biotechnology Information; NIBB, National Institute for Basic Biology; NJ, Neighbor-Joining; Paf1c, RNA polymerase II-associated factor 1 complex; RT-PCR, reverse transcription-PCR; SDG, SET DOMAIN GROUP; SUVH, SUV39 HOMOLOG; TRX, Trithorax; Ws, Wassilewskija.

## Introduction

Organisms are adapted to a wide range of environmental conditions by variation in genotype and phenotype. A well-studied example of such natural variation is the existence of both summer-annual and winter-annual habits in *Arabidopsis thaliana*. Summer-annual accessions flower rapidly and produce their offspring (i.e. seeds) in the same growing season in which they germinate. The summer-annual habit is likely to be advantageous in locations with a favorable growing environment that is sufficiently long and free of competition to complete the life cycle. Winter-annual accessions become established in the autumn season, overwinter, and then flower in the following spring. Winter-annual accessions typically have a strong requirement to undergo vernalization to prevent flowering in the autumn season in which they become established, thus ensuring spring flowering. Vernalization is the acquisition of competence to flower that results from exposure to the prolonged cold of winter (Chouard 1960). The winter-annual habit is advantageous in locations in which the autumn and early spring seasons provide the most favorable conditions for growth.

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In Arabidopsis, the vernalization requirement/winter-annual habit is typically established by active alleles of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Napp-Zinn 1979, Koornneef et al. 1994, Lee et al. 1994). *FLC* encodes a potent flowering repressor, and *FRI* is required for *FLC* to be transcribed to a level that effectively suppresses flowering (Michaels and Amasino 1999, Sheldon et al. 1999, Johanson et al. 2000).

Most commonly used 'lab strains' of Arabidopsis lack active *FRI* and/or *FLC* alleles, and exhibit rapid-flowering behavior in inductive long days. For instance, the Columbia (Col) wild type possesses a null allele of *fri* (Johanson et al. 2000) and, when *FRI* is introgressed into Col, it increases *FLC* transcription and causes a delay of flowering in non-vernalized plants (Lee and Amasino 1995). *FLC* activation due to the presence of *FRI* is accompanied by and requires histone modifications, such as tri-methylation of histone H3 at lysine 4 (H3K4me3) (Kim et al. 2005, Kim and Michaels 2006, Martin-Trillo et al. 2006, Choi et al. 2011, Jiang et al. 2011).

In eukaryotic cells, post-translational covalent modifications of core histones are involved in establishing the level of transcriptional activity of genes (reviewed in Li et al. 2007, Liu et al. 2010). A number of the histone modifications, including H3K4me2/me3, are associated with transcriptional activation, and there are also a range of histone modifications, such as H3K27me3, which are associated with transcriptional repression. Histone methylations at specific lysine residues are catalyzed by SET domain-containing proteins (Rea et al. 2000). The Arabidopsis genome encodes at least 47 proteins with a SET domain (Baumbusch et al. 2001, Springer et al. 2003, Ng et al. 2007). Based on the similarity to known H3K4-specific methyltransferases such as a *Drosophila* Trithorax (TRX) and yeast (*Saccharomyces cerevisiae*) Set1, five ARABIDOPSIS TRITHORAX (ATX1–ATX5) and two ATX-RELATED (ATXR3/SDG2 and ATXR7/SDG25) proteins have been proposed as H3K4-specific methyltransferases (Ng et al. 2007). Moreover, based on the similarities in amino acid sequence of the SET domains and the domain structures of the proteins, ATX1–ATX5 are likely to be TRX-class H3K4 methyltransferases, and ATXR7 appears to be the only ortholog of yeast Set1 (Alvarez-Venegas and Avramova 2001, Alvarez-Venegas et al. 2003, Avramova 2009). ATXR3 does not appear to belong to either the canonical TRX- or Set1-class methyltransferases, although the domain structure is similar to that of ATXR7 (Springer et al. 2003).

Among the putative Arabidopsis H3K4 methyltransferases, ATX1, ATX2 and ATXR7 have been shown to be involved in *FLC* activation and histone methylation (Pien et al. 2008, Saleh et al. 2008a, Berr et al. 2009, Jiang et al. 2009, Tamada et al. 2009). Mutations in these ATX genes partially suppress the delayed flowering caused by *FRI* (Pien et al. 2008, Tamada et al. 2009). *atx1 atx7* double mutants in particular exhibit decreased *FLC* expression and reduced levels of H3K4 mono-, di- and tri-methylation in the presence of *FRI*, indicating that these ATXs are required for *FLC* activation, presumably by catalyzing H3K4 methylation (Tamada et al. 2009).

However, we found that *atx1 atx2 atx7* does not completely suppress *FRI*-mediated increased *FLC* expression; thus, it is possible that other H3K4 methyltransferase(s) are required for *FLC* activation.

It was recently revealed that ATXR3/SDG2 is a major H3K4 tri-methyltransferase in Arabidopsis and is involved in many aspects of its development (Berr et al. 2010, Guo et al. 2010). For example, ATXR3 functions in the activation of *FLC* and flowering repression in the Col background (Guo et al. 2010). However, in the Col background, *FLC* expression is low due to lack of *FRI*, and *FLC* has a minor effect in flowering repression. It is thus of interest to analyze the ATXR3 function in *FLC* activation caused by an active allele of *FRI*. Furthermore, *FLC* activation cannot completely explain the role of ATXR3 function in flowering repression in non-inductive short days; thus, there must be another flowering repressor gene(s) that is activated by ATXR3.

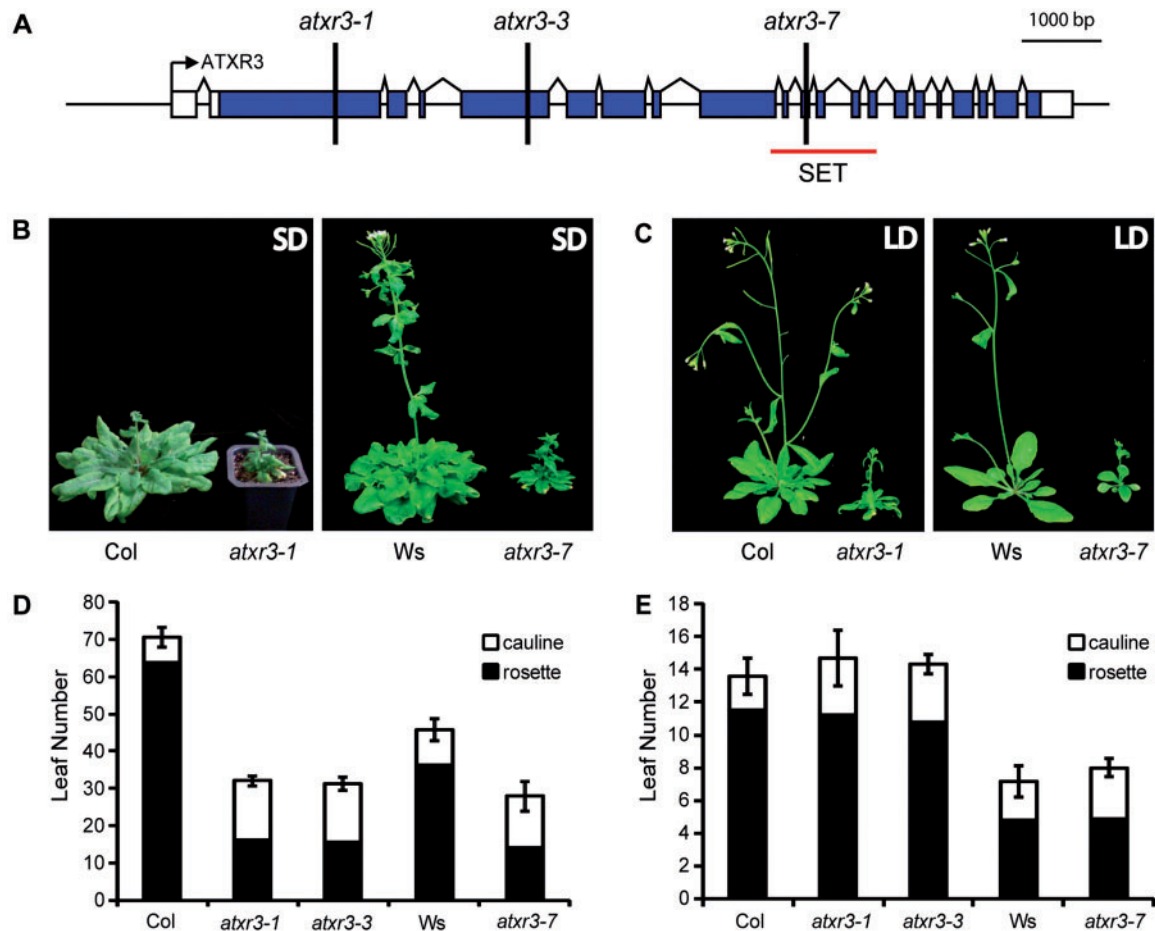
Here, we show that a lesion in ATXR3 suppresses the delayed flowering as well as the increased *FLC* expression caused by *FRI*. Levels of H3K4me3 were reduced around the transcription start site of *FLC* in the *FRI atxr3* mutant. In addition, rapid flowering of *atxr3* is epistatic to that of *atxr7*. These results indicate that ATXR3 influences transcriptional activation of *FLC* by catalyzing H3K4 tri-methylation of *FLC* chromatin in sequence with ATXR7 to establish the winter-annual habit of Arabidopsis. Loss of ATXR3 also results in reduced expression levels of other *FLC* clade members including *FLOWERING LOCUS M/MADS AFFECTING FLOWERING1* (*FLM/MAF1*) and *MAF5*, which at least partially accounts for the rapid flowering of *atxr3* in short days.

## Results

### A lesion in ATXR3 results in more rapid flowering than the wild type only in short days in the absence of *FRI*

To investigate the role(s) of ATXR3/SDG2 in Arabidopsis flowering, we characterized the phenotypes of three homozygous insertion mutants: *atxr3-1/sdg2-1* and *atxr3-3/sdg2-3* in the Col background (Tamada et al. 2009, Berr et al. 2010) (which were named *sdg2-2* and *sdg2-1*, respectively, by Guo et al. 2010) and *atxr3-7* in the Wassilewskija (Ws) background (Fig. 1A). The three insertions are in the second, fifth and 11th exon, and are predicted to prevent production of a protein with a functional SET domain. Loss of full-length ATXR3 transcripts has been previously confirmed for *atxr3-1* and *atxr3-3* (Berr et al. 2010, Guo et al. 2010).

All three (*atxr3-1*, *atxr3-3* and *atxr3-7*) alleles result in a similar pleiotropic phenotype that includes small plant size, as recently described (Guo et al. 2010), and increased cauline leaf number compared with the corresponding wild types (Fig. 1B–E). Guo et al. (2010) reported that *atxr3/sdg2* flowers more rapidly than the Col wild type in both long and short days.



**Fig. 1** ATXR3 gene structure and phenotypes of *atxr3* mutants. (A) ATXR3 gene structure and T-DNA insertion sites. An arrow, boxes and chevron lines indicate the transcription start site, exons and introns, respectively. Filled boxes represent the open reading frame regions, and open boxes indicate the untranslated regions. *atxr3-1* and *atxr3-3* are in the Col background, and *atxr3-7* is in the Ws background. (B and C) Representative plants of Col, *atxr3-1*, Ws and *atxr3-7* grown in short days (B, left panel, 13 weeks old; right panel, 12 weeks old) or in long days (C, left panel, 39 d old; right panel, 28 d old). *atxr3-1* and *atxr3-3* mutants have essentially identical phenotypes (data not shown). (D and E) Primary leaf number at flowering of Col, *atxr3-1*, *atxr3-3*, Ws and *atxr3-7* grown in short days (D) or in long days (E). Closed and open bars indicate rosette and cauline leaves, respectively. The averages of the results from at least nine plants are shown. Error bars indicate the SE.

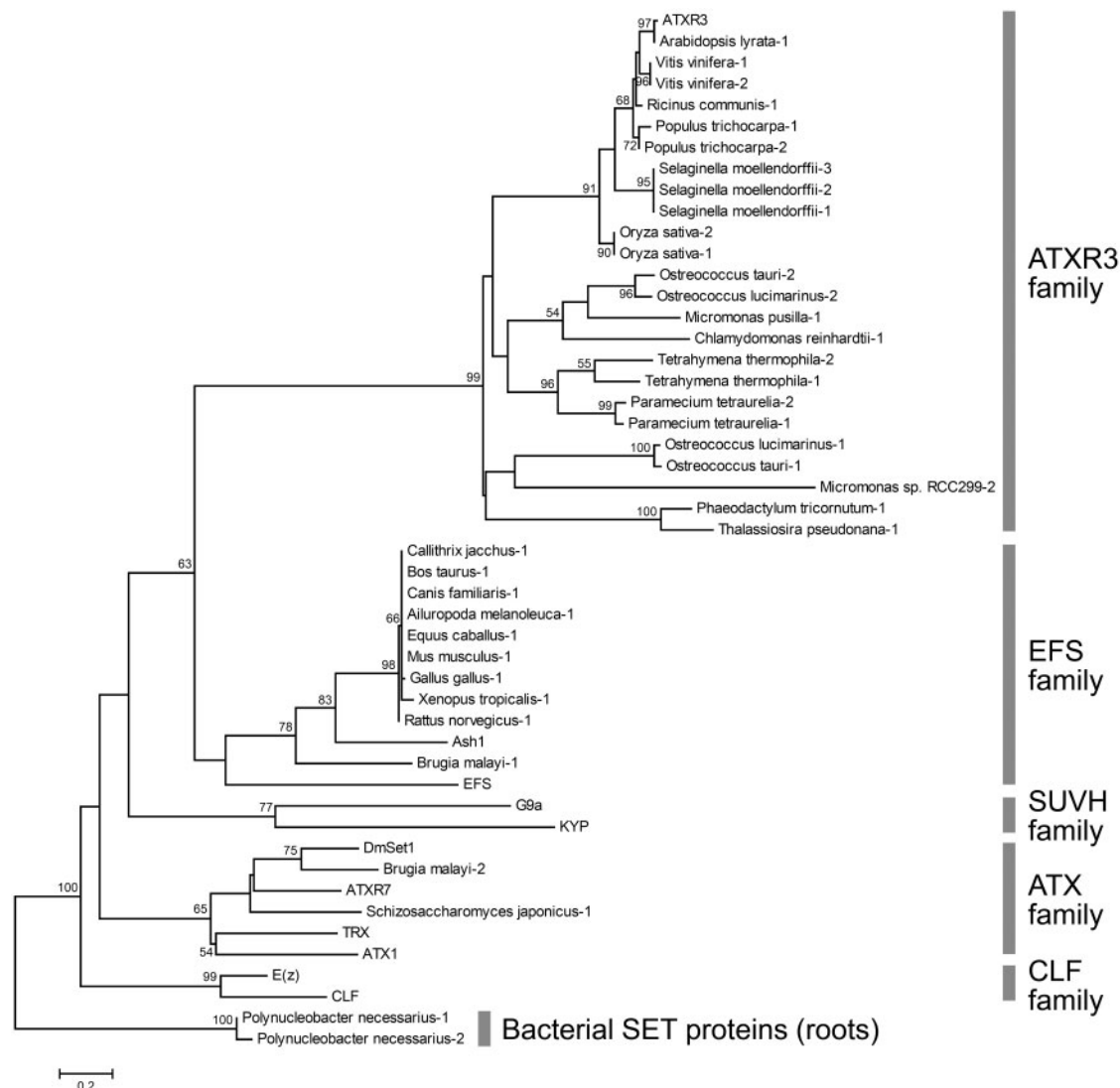
In our growth conditions, all three *atxr3* mutants flowered after forming fewer leaves than the corresponding wild types in short days (Fig. 1B, D); however, they exhibited flowering behavior similar to that of the wild types in long days (Fig. 1C, E). The growing temperature in our laboratory (21°C) is similar to that in the report by Guo et al. (2010) (22°C); thus, the discrepancies in the flowering time in long days is likely to be due to other differences in growth conditions such as light intensity.

In contrast to the lower number of leaves relative to the wild type that *atxr3* produces in short days, the actual number of days to flowering was greater in the *atxr3* mutants than in the wild types in both long and short days because the leaf initiation rate of *atxr3* mutants is substantially slower than that of the wild type (Supplementary Fig. S1A–C). Therefore, from a developmental perspective (leaf number), an *atxr3* lesion accelerates flowering in short days, but the overall time to

flowering is increased due to the substantially slower growth of the mutant. In another study, the leaf initiation rate of *atxr3* was reported to be comparable with that in the wild type before flowering (Berr et al. 2010). The difference in leaf initiation rate before flowering may be due to differences in growth conditions: we grew the plants in growth chambers, whereas a glasshouse was used in the study by Berr et al. (2010).

### ATXR3 belongs to a unique class of SET-domain proteins

We compared ATXR3 and proteins with a related amino acid sequence from a range of organisms in both bikont (Alveolata, Stramenopiles and Viridiplantae) and unikont (Fungi and Metazoa) groups (the sequences were obtained from BLASTp searches using ATXR3 as a query; Supplementary Table S1) with known SET-domain proteins from Arabidopsis



**Fig. 2** Phylogenetic relationships among ATXR3 and known histone methyltransferases. An ML tree was drawn using the SET domain sequences of known histone methyltransferases from *Arabidopsis* and *Drosophila*, bacterial SET proteins (*Polynucleobacter necessarius*-1, 2) and proteins that share similar amino acid sequences with ATXR3 (other proteins shown as Organism name-number) with PhyML (Guindon and Gascuel 2003, Guindon et al. 2010). KYP and G9a belong to the SUV39 HOMOLOG (SUVH) H3K9 methyltransferase family, and E(z) and CLF are in the H3K27 methyltransferase family. Numbers next to branches indicate bootstrap statistical support values for branches (1,000 replicates). Bootstrap values >50% are presented. The scale is the estimated branch length indicating the number of substitutions per site.

[ATX1, ATXR7, EARLY FLOWERING IN SHORT DAYS (EFS), CURLY LEAF (CLF) and KRYPTONITE (KYP)] and *Drosophila* {TRX, Set1 (DmSet1), Absent, small or homeotic discs 1 (Ash1), Enhancer of zeste [E(z)] and G9a}. As presented in the phylogenetic tree constructed from the SET domain sequences of these proteins (Fig. 2), ATXR3 and related proteins from bikonts formed a distinct clade, separate from those of unikonts and other known SET-domain proteins. Thus, ATXR3 appears to belong to a novel class of SET-domain proteins for which there was likely to be a founding member in the common ancestor of Alveolata, Stramenopiles and Viridiplantae. Alternatively, one cannot exclude the possibility that

ATXR3-like proteins were present in both the bikont and unikont groups, but were lost in the unikonts.

### The *atxr3* mutant suppresses *FRI*-dependent delayed flowering

The *Arabidopsis* winter-annual habit/vernalization requirement is conferred by the presence of active alleles of both *FRI* and *FLC* (Napp-Zinn 1979, Koornneef et al. 1994, Lee et al. 1994). *FRI*-mediated *FLC* activation is accompanied by an increase of H3K4me3 at *FLC* chromatin (Kim et al. 2005, Kim and Michaels 2006, Martin-Trillo et al. 2006, Choi et al. 2011, Jiang et al. 2011). To date, among a number of known/putative

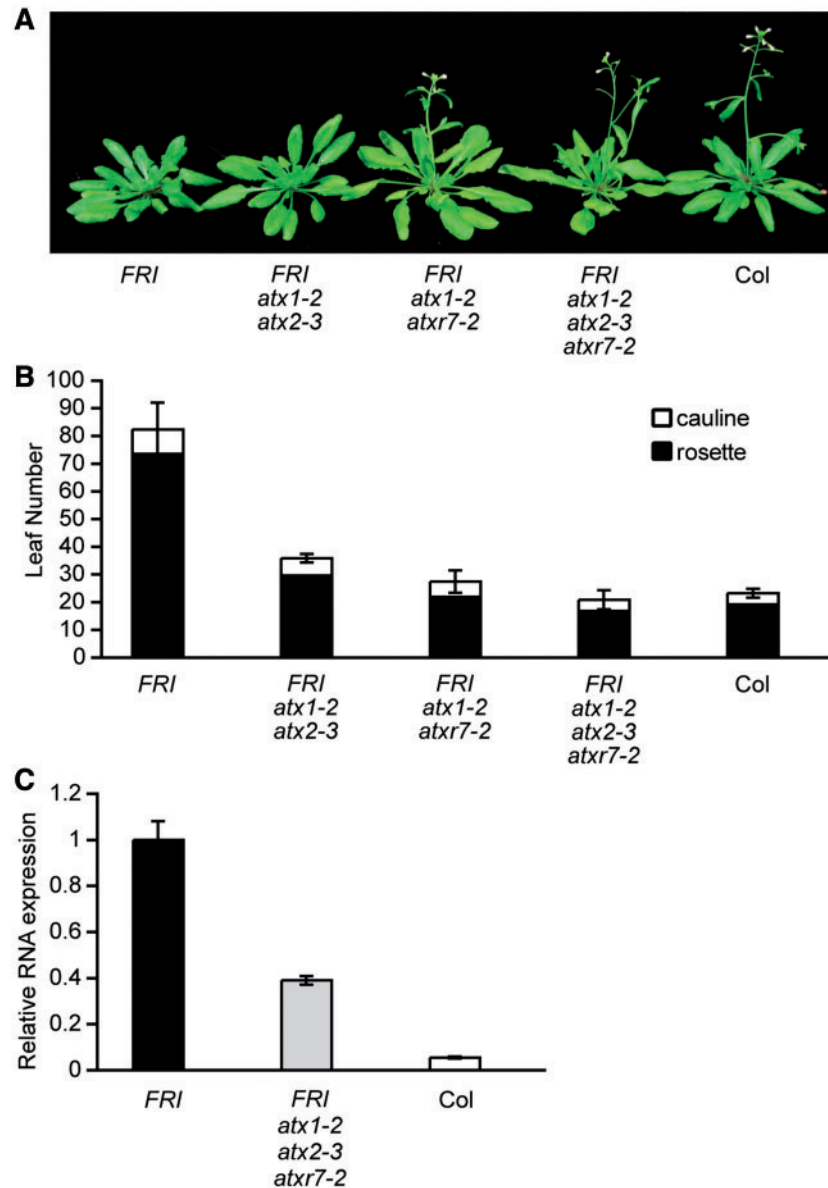


H3K4-specific methyltransferases, ATX1, ATX2 and ATXR7 are known to be required for *FRI*-mediated *FLC* activation (Pien et al. 2008, Tamada et al. 2009). However, the *FLC* mRNA levels in the *FRI atx1 atx2 atxr7* triple mutants are not as low as those in the Col wild type (i.e. a *fri* mutant) (Fig. 3), indicating that an additional H3K4 methyltransferase(s) could be involved in *FLC* activation. To evaluate the possible involvement of ATXR3 in *FLC* activation, an active *FRI* locus was introgressed into the *atxr3* mutant and the flowering time was analyzed. The *atxr3* mutation significantly suppressed the

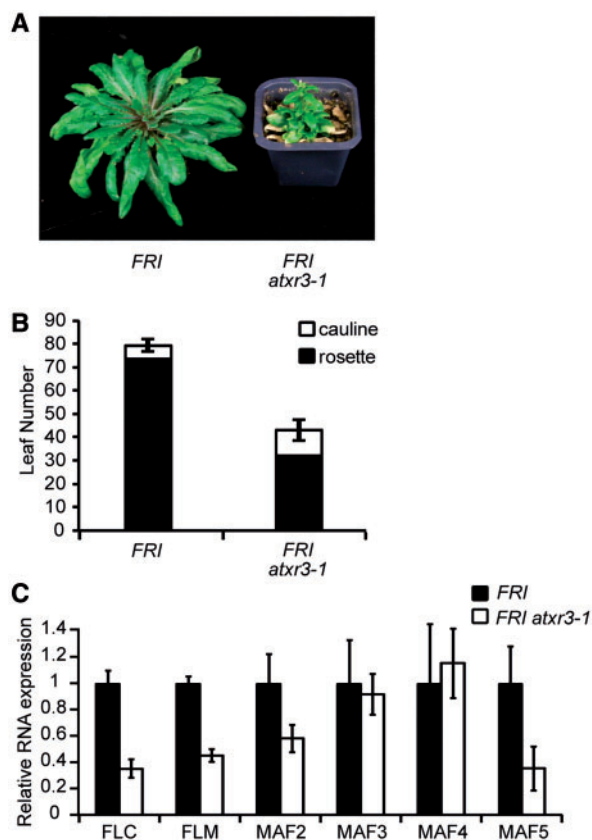
*FRI*-induced delayed flowering (Fig. 4A, B). Thus, ATXR3 is also required for *FRI*- and *FLC*-dependent delayed flowering.

### ATXR3 is required for proper expression of *FLC* and *FLC* clade genes

To assess whether ATXR3 is involved in setting the level of *FLC* expression in the presence of *FRI*, *FLC* mRNA levels were analyzed in *FRI atxr3* and *FRI-Col* (Col into which *FRI* has been introgressed; Lee and Amasino 1995). *FLC* mRNA levels in *FRI*



**Fig. 3** Phenotypes of the *atx1 atx2 atxr7* triple mutant. (A) Representative plants of *FRI-Col*, *FRI atx1-2 atx2-3*, *FRI atx1-2 atxr7-2*, *FRI atx1-2 atx2-3 atxr7-2* and Col (7 weeks old) grown in long days. At this stage, half of the *FRI atx1-2 atx2-3* plants started to flower, but none of the *FRI-Col* plants had flowered. (B) Primary leaf number at flowering of *FRI-Col*, Col and *atx* multiple mutants grown in long days. Closed and open bars indicate rosette and cauline leaves, respectively. Error bars indicate the SD. (C) Real-time PCR was performed to analyze expression levels of *FLC* in *FRI-Col*, *FRI atx1-2 atx2-3 atxr7-2* and Col. RNA was isolated from whole 10-day-old seedlings grown in continuous light. The averages of the results from three different biological replicates are shown. Each experiment was normalized to *ACTIN7* (*ACT7*) expression. Error bars indicate the SE.



**Fig. 4** Phenotypes of *atxr3* in a *FRI-Col* background. (A) Representative plants of *FRI-Col* and *FRI atxr3-1* (12 weeks old) grown in long days. (B) Primary leaf number at flowering of *FRI-Col* and *FRI atxr3-1* grown in long days. Closed and open bars indicate rosette and cauline leaves, respectively. The averages of the results from at least 12 plants are shown. Error bars indicate the SE. (C) Real-time PCR was performed to analyze the expression of *FLC* and the *FLC*-clade genes in *FRI-Col* (filled bars) and *FRI atxr3-1* (open bars). Almost fully expanded rosette leaves that look vital and completely green were sampled from several 6-week-old plants. Plants had not flowered yet at this stage. The averages of the results from four different biological replicates are shown. The data from each experiment were normalized to *ACT7* expression. Error bars indicate the SE.

*atxr3* were lower compared with those in the wild type *FRI-Col* ( $P < 0.05$ ; Fig. 4C). Thus, the earlier developmental stage when flowering occurs in *FRI atxr3* relative to *FRI-Col* is likely to be due, at least in part, to a reduction of *FLC* expression.

A reduction of *FLC* expression alone has only a minor effect in flowering without an active *FRI* allele (Michaels and Amasino 2001); thus, rapid flowering of *atxr3* in short days in the *fri* (i.e. Col and Ws) backgrounds (Fig. 1; Guo et al. 2010) cannot be explained by only reduced *FLC* expression. Most of the histone modifiers that play a role in the *FLC* activation are also involved in the transcriptional activation of other *FLC* clade members, such as *FLM/MAF1* and/or *MAF2-5* (Kim et al. 2005, Cao et al. 2008, Xu et al. 2008, Gu et al. 2009, Schmitz et al. 2009, Tamada et al. 2009, Xu et al. 2009). In fact, *FRI atxr3-1* exhibited reduced levels of *FLM* and *MAF5* transcripts relative

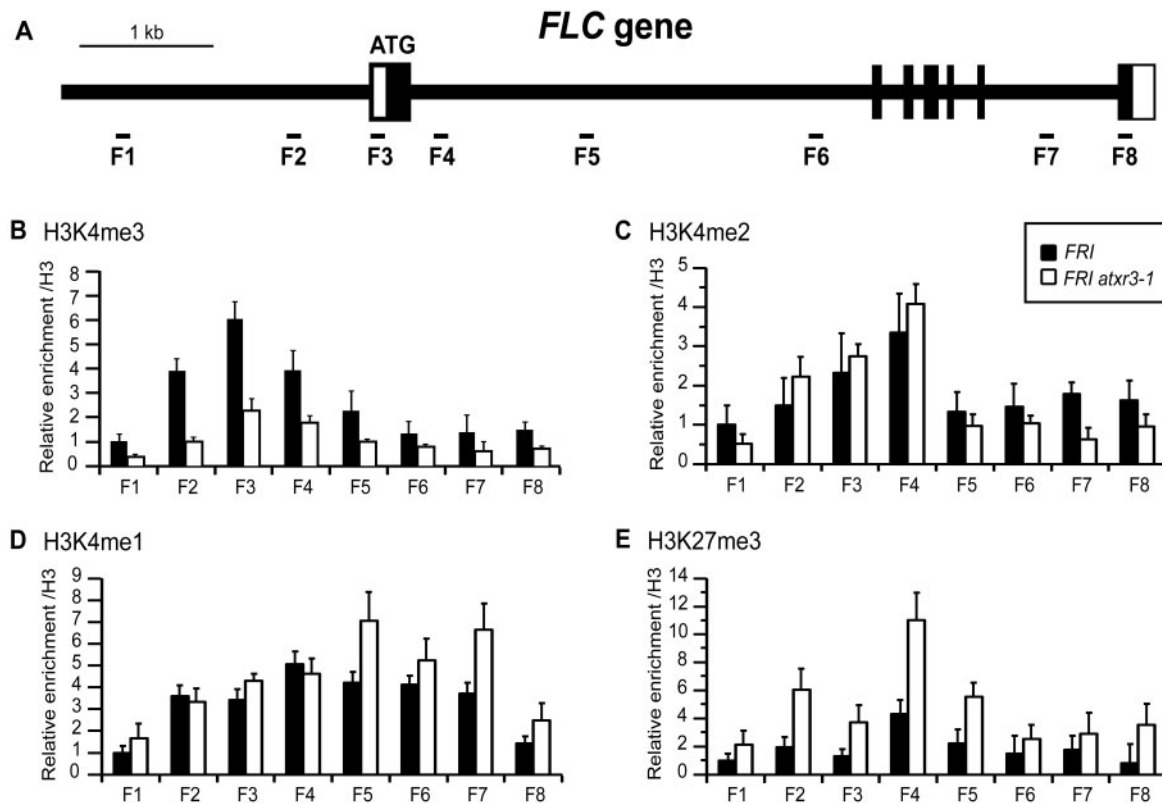
to *FRI-Col* wild type ( $P < 0.05$ ; Fig. 4C). However, no significant change was observed for *MAF2*, *MAF3* and *MAF4* expression in the mutant ( $P > 0.05$ ; Fig. 4C). *FLM* is known to be required for the photoperiod-dependent repression of flowering (Ratcliffe et al. 2001, Ratcliffe et al. 2003, Scortecci et al. 2003). Reduced expression of *FLM* and *MAF5*, in addition to *FLC*, at least partially accounts for the attenuation of the photoperiod-dependent delay of flowering in *atxr3*.

### Loss of ATXR3 affects histone modifications at *FLC* chromatin

As discussed above, we have established that *ATXR3* is required for proper levels of *FLC* expression in the presence of *FRI* (Fig. 4). *ATXR3* encodes a member of a novel class of SET domain proteins (Fig. 2) which is a major H3K4-specific methyltransferase (Berr et al. 2010, Guo et al. 2010). Although *ATXR3* catalyzes all mono-, di- and tri-methylation of H3K4 in vitro, only global reduction of tri-methylation (H3K4me3) was observed in *atxr3* in vivo (Berr et al. 2010, Guo et al. 2010). The effects of an *atxr3* lesion on global levels of H3K4me1/me2 are different in the reports by Guo et al. (2010) and Berr et al. (2010): Guo et al. observed increased levels of global H3K4me2 in *atxr3/sdg2*, while Berr et al. obtained slightly decreased H3K4me2 and increased H3K4me1 levels in the mutant. It was of interest for us to evaluate the effect of loss of *ATXR3* function on H3K4 methylations at *FLC* in a *FRI* background using chromatin immunoprecipitation (ChIP) with anti-H3K4me3, H3K4me2 or H3K4me1 antibodies (Fig. 5A).

Levels of an active mark, H3K4me3, were enriched particularly around the transcriptional start site of the active *FLC* locus in *FRI-Col* (Fig. 5B; similar results have been reported in He et al. 2004, Kim et al. 2005, Oh et al. 2008, Tamada et al. 2009). However, loss of *ATXR3* function resulted in reduced H3K4me3 levels across the entire *FLC* genomic region (Fig. 5B), and the most significant reduction was around the transcriptional start site relative to *FRI-Col* wild type ( $P < 0.05$ ; Fig. 5B: F2-4). The reduced H3K4me3 levels at *FLC* in *FRI atxr3-1* correlate with the reduction of *FLC* transcription in the mutant (Fig. 4C). In *FRI-Col* wild type, the pattern of H3K4me2 is similar to that of H3K4me3 (Fig. 5C). However, unlike the case for H3K4me3, the *atxr3* mutation did not result in a reduction of H3K4me2 around the transcriptional start site (Fig. 5C: F2-4), although there was a slight reduction of H3K4me2 in the transcribed region of *FLC* (Fig. 5C: F5-8). Finally, there was no significant reduction of the H3K4me1 level across the *FLC* region in the *atxr3* mutant (Fig. 5D); in fact, there is a slight increase in H3K4me1 in the transcribed region of *FLC* (Fig. 5D: F5-7). Overall, the effects of an *atxr3* lesion on the H3K4 methylation levels at *FLC* are similar to those on global H3K4 methylation observed by Berr et al. (2010).

Another chromatin modification, H3K27me3, is involved in the transcriptional repression of *FLC*. H3K27me3 is highly enriched at *FLC* when its expression is perturbed (see the



**Fig. 5** Histone methylation statuses at *FLC* in *FRI*-Col and the *FRI atxr3-1* mutant. (A) Schematic model of primer positions at the *FLC* locus. (B–E) Levels of H3K4me3 (B), H3K4me2 (C), H3K4me1 (D) and H3K27me3 (E) at *FLC*. The x-axis and y-axis indicate the primer number described in A and relative levels of modifications, respectively. Relatively young rosette leaves were sampled from several 4-week-old plants. Plants had not flowered at this stage. The averages of the results from at least two different biological replicates are shown. The data from each experiment were normalized to either *ACT7* (H3K4 methylations) or *AGAMOUS* (*AG*) (H3K27me3) and then were further normalized to the total histone H3 ChIP. Error bars indicate the SE.

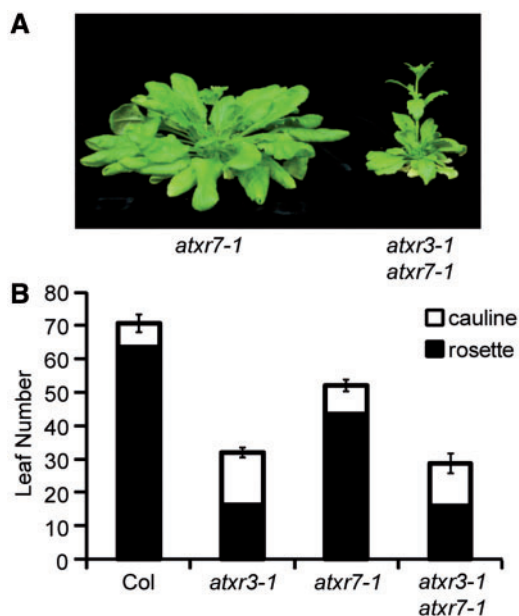
review in this special issue; Buzas et al. 2012). For example, loss of the *ATXR7* function causes reduced *FLC* mRNA levels and increased H3K27me3 levels at *FLC* (Tamada et al. 2009). However, another H3K4 methyltransferase, *ATX1*, is the only known exception; both *FLC* expression and the H3K27me3 levels at *FLC* are lower in *atx1* than in the wild type (Pien et al. 2008). Therefore, it is of interest to examine whether a novel class H3K4 methyltransferase, *ATXR3*, functions like *ATX1* or *ATXR7* for H3K27me3. In *FRI atxr3-1*, we observed an increased level of H3K27me3 relative to *FRI*-Col wild type across the entire *FLC* locus with ChIP (Fig. 5E). This indicates that *ATXR3* functions like *ATXR7* for H3K27me3, and *ATXR3*-mediated H3K4 tri-methylation and the concomitant transcription, which is enhanced by H3K4me3, acts to antagonize H3K27 tri-methylation at *FLC* (Buzas et al. 2011, Schmitges et al. 2011).

### In short days *atxr3* is epistatic to *atxr7* for flowering time whereas *atxr7* is epistatic to *atxr3* with respect to growth rate

Although the SET domain of *ATXR3* is diverged from those of other known H3K4 methyltransferases (Fig. 2), the overall

protein structure of *ATXR3* is similar to that of *ATXR7*: both *ATXR3* and *ATXR7* share GYF domains in the N-terminus (Springer et al. 2003). In addition, like *ATXR3*, *ATXR7* is required for repression of flowering both in short days and in the presence of *FRI* (Tamada et al. 2009). To investigate the genetic interaction between *ATXR3* and *ATXR7* in flowering, an *atxr3-1 atxr7-1* double mutant was created and analyzed for flowering time in short days relative to the wild type and the single mutants. Consistent with our previous findings, the *atxr7-1* single mutant flowered after forming ~50 leaves in short days (Fig. 6A, B). Interestingly, the *atxr3-1 atxr7-1* double mutant flowered after forming ~30 leaves, which is similar to the leaf number at flowering of the *atxr3-1* single mutant (Fig. 6B). Thus, a lesion in *ATXR7* does not cause any further developmental acceleration of flowering when combined with a lesion in *ATXR3*, indicating that the role of *ATXR7* is largely dependent on *ATXR3* with respect to short day flowering.

We also monitored the days to the appearance of a visible inflorescence of the *atxr3-1 atxr7-1* double mutant and each single mutant. As mentioned earlier, *atxr3-1* exhibited an extended time to flower due to the slower growth rate relative to wild-type Col (Supplementary Fig. S1A–C). The *atxr7-1*



**Fig. 6** Phenotypes of the *atxr3 atxr7* double mutant. (A) Representative *atxr7-1* and *atxr3-1 atxr7-1* plants (9 weeks old) grown in short days. (B) Primary leaf number at flowering of Col, *atxr3-1*, *atxr7-1* and *atxr3-1 atxr7-1* grown in short days. Closed and open bars indicate rosette and cauline leaves, respectively. The averages of the results from at least nine plants are shown. Error bars indicate the SE.

single mutant has an essentially wild-type growth rate and thus required less time to flower than the *atxr3-1* single mutant (Supplementary Fig. S1B, C). Notably, in the *atxr3 atxr7* double mutant, the extended time to flower of *atxr3* was suppressed; the double mutant had a growth rate similar to that of the *atxr7-1* single mutant (Supplementary Fig. S1B, C). Therefore, *atxr7* is epistatic to *atxr3* with respect to the growth rate, unlike the epistatic relationship for the developmental acceleration of flowering described above.

## Discussion

The level of gene expression is often correlated with the levels of the H3K4me3 enrichment around transcription and translation start sites. It has been previously shown that certain Arabidopsis H3K4 methyltransferases, ATX1 and ATX2 in the TRX class and ATXR7 in the Set1 class, function in the *FLC* activation and flowering repression caused by *FRI* (Pien et al. 2008, Tamada et al. 2009). In the work reported herein, we have shown that ATXR3/SDG2, which is a member of a novel class of H3K4-specific methyltransferases (Fig. 2; Berr et al. 2010, Guo et al. 2010), also plays a role in *FRI*-dependent *FLC* activation. An ATXR3 lesion suppresses the *FLC* activation and the delayed flowering of the *FRI*-containing line *FRI*-Col in long days (Fig. 4). The reduced *FLC* expression in *atxr3* mutants correlates with decreased H3K4me3 levels at the *FLC* locus (Fig. 5B). Collectively, three divergent types of H3K4-specific

methyltransferases encoded by four genes (TRX-class ATX1 and ATX2, Set1-class ATXR7 and ATXR3) appear to be required for full levels of H3K4me3 at *FLC* chromatin and *FLC* expression in the presence of an active *FRI* allele, and thus for the winter-annual habit of Arabidopsis.

In contrast to H3K4me3, minor differences were found for the H3K4me1/me2 levels between *FRI atxr3* and the wild type in the gene body of *FLC*: the H3K4me2 levels are reduced and the H3K4me1 levels are increased in *FRI atxr3* compared with the wild type (Fig. 5C, D). These data suggest that H3K4me3, but not H3K4me1/me2, is involved in the *FLC* activation. We also found that, in *FRI atxr3*, the levels of the repressive epigenetic mark, H3K27me3, are increased compared with the *FRI*-Col wild type (Fig. 5E). Thus, ATXR3 appears to be part of an antagonistic system of methyltransferases that set the proper level of *FLC* expression by establishing and maintaining a balance between H3K4 and H3K27 methyltransferase activities (reviewed in Buzas et al. 2012). The antagonistic nature of H3K4 and H3K27 methylations at target loci has been observed in a range of eukaryotic systems (Papp and Muller 2006, Suganuma and Workman 2008, Schmitges et al. 2011).

Loss of the ATXR3 function also suppresses the delayed flowering in short days of lines that lack *FRI* (Fig. 1; Guo et al. 2010). This suppression of delayed flowering is associated with reduced expression of both *FLC* (Guo et al. 2010) and *FLC* clade members *FLM* and *MAF5* (Fig. 4C). Thus, ATXR3 participates in the repression of flowering by maintaining the activated state of *FLC* as well as certain *FLC*-clade genes that are not affected by *FRI*. The expression of *FLM* and *MAF5* was not increased in *atxr3* in a previous microarray analysis (Guo et al. 2010). This discrepancy may be caused by the differences in the developmental stage of the plant material (we used almost fully expand rosette leaves compared with entire 12-day-old seedlings) and/or in methods [real-time reverse transcription-PCR (RT-PCR) vs. microarray].

Although ATXR3 and ATXR7 have divergent SET domains (Fig. 2), they have a similar domain organization, with GYF domains in the N-terminus (Springer et al. 2003), and share similar roles in flowering repression in the presence of *FRI* and in non-inductive short days (Figs. 1, 3; Berr et al. 2009, Tamada et al. 2009, Guo et al. 2010). It was therefore of interest to evaluate their possible relationship by characterizing the *atxr3 atxr7* double mutant. The developmental acceleration of flowering is stronger in the *atxr3* single mutant than in the *atxr7* single mutant and, interestingly, in the *atxr3 atxr7* double mutant there is not any additional flowering acceleration compared with the *atxr3* single mutant (Fig. 6B). Thus, *atxr3* is epistatic to *atxr7* with respect to a developmental measure of flowering. This epistatic relationship indicates that the ability of ATXR7 to participate in activation of *FLC* and other flowering repression genes (such as *FLC* clade genes) is largely dependent on ATXR3.

How do ATXR3 and ATXR7 function together in *FLC* activation? One possibility is that they have different methyltransferase activities and together establish the H3K4me3 mark



at *FLC*. As described above, the *atxr3* lesion in a *FRI* background results in reduced H3K4me2/me3 levels and increased H3K4me1 levels at *FLC* (Fig. 5). In contrast, the levels of all mono-, di- and tri-methylations of H3K4 are decreased in *FRI atxr7* relative to those in *FRI-Col* (Tamada et al. 2009). This difference may be accounted for by the 'Phe/Tyr switch' model; a tyrosine at a particular amino acid residue in the SET domain is considered to indicate that the protein has mono- and/or di-methyltransferase activities, whereas a phenylalanine indicates di- and/or tri-methyltransferase activities (Collins et al. 2005, Couture et al. 2008). ATXR3 has a phenylalanine in the key position and is thus predicted to have di- and tri-methyltransferase activities (Guo et al. 2010) and ATXR7 has a tyrosine and thus mono- and di-methyltransferase activities. In this case, the increased levels of H3K4me1 in *FRI atxr3* are likely to be due to the attenuation of H3K4 di- and tri-methylation activities, and reduced levels of H3K4me3 in *FRI atxr7* could result from decreased H3K4 mono- and di-methylation activities as such activities are required to produce the substrate for tri-methylation. Given that the *FLC* activation is associated with the H3K4me3 enrichment around the transcription and translation start sites, di- and tri-methyltransferase activity of ATXR3, together with mono- and di-methyltransferase activities of ATXR7, are required for full *FLC* expression. However, we should also mention that ATXR3 is reported to possess all mono-, di- and tri-methyltransferase activities in vitro (Guo et al. 2010). Further biochemical analyses for ATXR3 and ATXR7 will be interesting. Another possibility is that GYF domains in ATXR3 and ATXR7 together play a role in the expression of *FLC* and other flowering time genes (Springer et al. 2003). The GYF domain has been reported to interact with proline-rich sequences in proteins involved in splicing and mRNA export from the nucleus (reviewed in Kofler and Freund 2006). The GYF domains in ATXR3 and ATXR7 may function together in the splicing and mRNA export of the transcripts of *FLC* and other flowering time genes.

The epistatic relationship between *atxr3* and *atxr7* is reminiscent of that between *early flowering7 (elf7)* and *atxr7* in which *elf7* is epistatic to *atxr7* (Tamada et al. 2009). ELF7 is a component of the RNA polymerase II-associated factor 1 complex (Paf1c), and this epistatic relationship is consistent with a model in which ELF7 may function in ATXR7 recruitment (Krogan et al. 2003, Tamada et al. 2009). This raises the possibility that there exists a functional link between ATXR3 and Paf1c. It is also interesting to note that the function of Paf1c and ATXR7 is largely independent of that of EFS, which appears to possess di- and tri-methyltransferase activities on H3K4 and H3K36 (Ko et al. 2010), based on the previous observation of transgressive phenotypes in *atxr7 efs* or *elf7 efs* double mutants (Tamada et al. 2009). Based on these epistatic relationships, ATXR3 function is also likely to be independent of EFS. In support of this model, it was recently shown that the genome-wide expression pattern in *atxr3* is not correlated with that of *atx1* or *efs* (Guo et al. 2010). From the results we and other groups

have obtained, we propose a hypothetical model of *FLC* activation by the H3K4 methyltransferases and Paf1c (Fig. 7).

In addition to the epistasis in the flowering time regulation, we also observed the epistatic relationship in the growth rate regulation between ATXR3 and ATXR7: the slower growth rate in *atxr3* vs. that in the wild type was suppressed by *atxr7*, suggesting that *atxr7* is epistatic to *atxr3* with respect to growth rate (Supplementary Fig. S1). If the gene(s) responsible for the slower growth rate is directly regulated by both ATXR3 and ATXR7 as in the case of *FLC*, ATXR3 and ATXR7 may have opposite roles in the transcription (i.e. ATXR7 or ATXR3 may have the unexpected function of transcriptional repression). Another possibility is that the gene(s) responsible may not be the direct target of ATXR3 and/or ATXR7. In fact, both Berr et al. (2010) and Guo et al. (2010) found a significant number of genes (two out of five genes and 22 out of 30 genes, respectively) with the microarray and individual CHIP analyses, for which changes in the mRNA levels are not correlated with those in the H3K4me3 levels. These genes are thought to be the indirect targets of ATXR3 (Berr et al. 2010, Guo et al. 2010). Isolation of the responsible gene(s) will be necessary to understand the epistasis in the growth rate regulation between ATXR3 and ATXR7.

## Materials and Methods

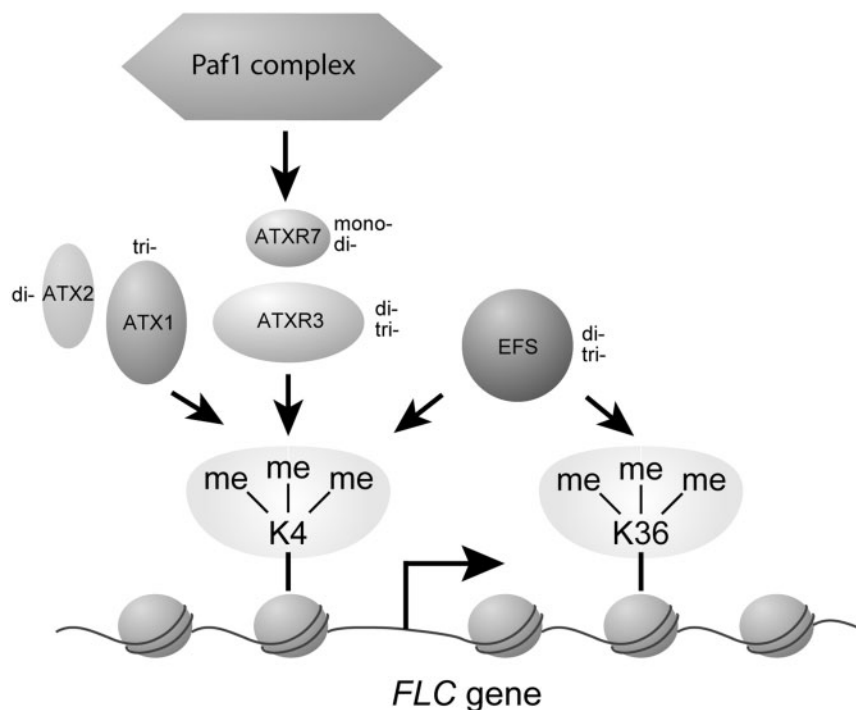
### Plant materials and growth conditions

*FRI-Col* is the *Col* genetic background into which an active *FRI* locus of accession San feliu-2 was introgressed (Lee and Amasino 1995). Seeds of *atxr3-1* (WiscDsLox361D10) and *atxr3-3* (SALK\_021008) were obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al. 2003, Woody et al. 2007). *atxr3-7* (FLAG\_224A03/G01) seeds were obtained from the National Institute for Agricultural Research (INRA) (Samson et al. 2002).

Plants were grown at 21°C in long days (16 h light/8 h dark), short days (8 h light/16 h dark) or continuous light from cool-white fluorescent tubes [photosynthetic photon flux density (PPFD) approximately 60–70  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ]. Seeds were incubated in water at 4°C for 2 d and were then directly sown on the soil surface (Sun-Gro; MetroMix360), or incubated on agar-solidified medium containing 0.65 g l<sup>-1</sup> Peters Excel 15-5-15 fertilizer (Grace Sierra) at 4°C for 2 d, and were transferred on the same agar medium to either long days, short days or continuous light.

### Phylogenetic analysis and multiple alignments

BLASTp searches were performed using the entire or the SET domain sequence of ATXR3 as a query on the National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). SET domain sequences of known histone methyltransferases, bacterial SET proteins (*Polynucleobacter necessarius*-1, 2) that were used for the roots, and the proteins from the BLASTp searches



**Fig. 7** Model of a possible relationship between chromatin-modifying complexes that are involved in *FLC* activation. Our results indicate that the function of ATXR7 in flowering regulation is dependent on ATXR3. Relationships among ATXR7, ATX1, Paf1 complex and EFS were described previously (Tamada et al. 2009). Mono-, di- and tri- next to the factors indicate the predicted or examined activities of H3K4 methyltransferases. ATXR3 has phenylalanine and ATXR7 has tyrosine at the key residue for the methyltransferase activities, suggesting that ATXR7 catalyzes mono- and di-methylation of H3K4, and that ATXR3 catalyzes di- and tri-methylation to activate *FLC*. The activities of other H3K4 methyltransferases were described in previous studies (Pien et al. 2008, Saleh et al. 2008b, Guo et al. 2010, Ko et al. 2010, Ko et al. 2010). EFS contributes to H3K4 methylations at *FLC* most probably only in the presence of *FRI* (Kim et al. 2005, Zhao et al. 2005, Xu et al. 2008, Ko et al. 2010). K4, Lys4 of histone H3 (H3K4); K36, Lys36 of histone H3 (H3K36); me, methyl groups on lysine residues.

(**Supplementary Table S1**) were obtained using the CDD program on the NCBI website (Marchler-Bauer et al. 2009). Protein models from organisms in Alveolata, Stramenopiles and Viridiplantae that lack a SET domain or that are too short to include in the phylogenetic analyses were omitted from the analyses. The SET domain sequences were then aligned using the linsi program on the mafft alignment application (Kato et al. 2005; <http://mafft.cbrc.jp/alignment/software/>) and the alignment of sequences was refined manually using MEGA5 (Tamura et al. 2011; <http://www.megasoftware.net/>). From the alignment, a maximum likelihood (ML) tree was obtained with PhyML (Guindon and Gascuel 2003, Guindon et al. 2010; <http://atgc.lirmm.fr/phyml/>) using a Neighbor-Joining (NJ) tree and 200 random trees as initial trees with eight substitution categories, Best of NNI and SPR moves, and a bootstrap analysis (1,000 replicates). We adopted the default settings for the other parameters.

### Molecular analyses

Total RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNAs were synthesized from 3 µg of total RNA by using M-MLV Reverse Transcriptase (Promega) according to the

manufacturer's recommendations. Real-time PCR was performed on a 7500 Fast Real-time PCR system (Applied Biosystems) using a DyNAmo HS SYBR Green qPCR kit (Finnzymes) according to the manufacturer's protocol. The PCR conditions were one cycle of 15 min at 95°C, and 45 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C, followed by the dissociation stage as recommended by the manufacturer. Primers used to amplify the cDNAs are listed in **Supplementary Table S2**. All real-time RT-PCR data shown herein are the averages of the results from at least three different biological replicates, and were normalized to *ACT7* expression.

ChIP was performed as previously described (Johnson et al. 2002, Gendrel et al. 2005). Antibodies used in this study were purchased from Millipore: histone H3 (06-755), H3K4me3 (07-473), H3K4me2 (07-030), H3K4me1 (07-436) and H3K27me3 (07-449). Real-time PCR was performed on a StepOnePlus Real-time PCR system (Applied Biosystems) using SYBR premix Ex Taq (TAKARA BIO INC.) according to the manufacturer's protocol. The PCR conditions were one cycle of 30 s at 95°C, and 45 cycles of 5 s at 95°C, 15 s at 55°C and 30 s at 70°C, followed by the dissociation stage as recommended by the manufacturer. Primers used to detect the enrichment are listed in **Supplementary Table S2**. All real-time

ChIP-PCR data shown herein are the averages of the results from at least two different biological replicates, and were normalized to the level of either ACT7 or AG enrichment.

### Accession numbers

Accession numbers of genes in this article are as follows: At2g31650 (ATX1), At1g05830 (ATX2), At3g61740 (ATX3), At4g27910 (ATX4), At5g53430 (ATX5), At4g15180 (ATXR3/SDG2), At5g42400 (ATXR7/SDG25), At4g00650 (FRI), At1g77300 (EFS), At5g10140 (FLC), At1g77080 (FLM), At5g65050 (MAF2), At5g65060 (MAF3), At5g65070 (MAF4), At5g65080 (MAF5), At5g09810 (ACT7), At4g18960 (AG), At2g23380 (CLF) and At5g13960 (KYP). Accession numbers of the genes used in the phylogenetic analysis are described in **Supplementary Table S1**. Stock numbers of the T-DNA insertion lines described in this manuscript are as follows: WiscDsLox361D10 (*atxr3-1*), SALK\_021008 (*atxr3-3*), FLAG\_224A03/G01 (*atxr3-7*) and SALK\_149692 (*atxr7-1*).

### Supplementary data

**Supplementary data** are available at PCP online.

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