Plant Science 208 (2013) 20–27

Contents lists available at SciVerse ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Genetic identification of a novel locus, *ACCELERATED FLOWERING 1* that controls chromatin modification associated with histone H3 lysine 27 trimethylation in *Arabidopsis thaliana*

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ARTICLE INFO

Article history:
Received 10 February 2013
Received in revised form 11 March 2013
Accepted 12 March 2013
Available online 21 March 2013

Keywords:
Afl1-1D
Early flowering
H3K27me3
Chromatin modification
Arabidopsis thaliana

ABSTRACT

Flowering on time is a critically important for successful reproduction of plants. Here we report an early-flowering mutant in *Arabidopsis thaliana*, *accelerated flowering 1-1D* (*afl1-1D*) that exhibited pleiotropic developmental defects including semi-dwarfism, curly leaf, and increased branching. Genetic analysis showed that *afl1-1D* mutant is a single, dominant mutant. Chromosomal mapping indicates that *AFL1* resides at the middle of chromosome 4, around which no known flowering-related genes have been characterized. Expression analysis and double mutant studies with late flowering mutants in various floral pathways indicated that elevated *FT* is responsible for the early-flowering of *afl1-1D* mutant. Interestingly, not only flowering-related genes, but also several floral homeotic genes were ectopically overexpressed in the *afl1-1D* mutants in both *FT*-dependent and -independent manner. The degree of histone H3 Lys27-trimethylation (H3K27me3) was reduced in several chromatin including *FT*, *FLC*, *AG* and *SEP3* in the *afl1-1D*, suggesting that *afl1-1D* might be involved in chromatin modification. In support, double mutant analysis of *afl1-1D* and *lhp1-4* revealed epistatic interaction between *afl1-1D* and *lhp1-4* in regard to flowering control. Taken together, we propose that *AFL1* regulate various aspect of development through chromatin modification, particularly associated with H3K27me3 in *A. thaliana*.

1. Introduction

Flowering, the switch from vegetative to reproductive growth, is one of the most critical developmental changes that ensure successful reproduction of higher plants. For timely flowering, plants should integrate environmental cues and endogenous developmental programs. For the past decades, a number of genes have been identified to control when to flower, and they have been categorized into four distinct and interacting genetic pathways: photoperiod, vernalization, autonomous and gibberellin (GA)-dependent pathways in *Arabidopsis* [1–4]. Recent elegant studies revealed the existence of key floral integrators, such as *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS C* (*FLC*), *SUPPRESSOR OF CO1* (*SOC1*), and *LEAFY* (*LFY*), of which expression or activity are the converged targets of the flowering control [2,5–9].

Most well characterized integrators of flowering pathways is *FT* [10]. In photoperiod dependent pathway, the coincidence of light-exposure during the peak expression of *CONSTANS* (*CO*) activates expression of *FT* in the leaf. Other several DNA-binding transcription factors including *CRY2 INTERACTING BASIC HELIX LOOP HELIX 1/5* (*CIB1/5*), *FLOWERING LOCUS C* (*FLC*), and *SHORT VEGETATIVE PHASE* (*SVP*) have been characterized to regulate *FT* expression in response to light-quality, autonomous pathway/vernalization, and ambient temperature, respectively [11–15]. The induced FT protein moves from leaf to meristem, where it interacts with a bZIP transcription factor, *FLOWERING LOCUS D* (*FD*) to activate floral meristem identity genes [16,17].

In addition to the transcriptional regulators, recent studies highlight the critical roles of epigenetic control on floral integrators [18,19]. Expression of *FT* is under control of various histone modifications. For example, The *FT* locus are marked with both repressive histone H3 Lys27 tri-methylation (H3K27me3) and active histone H3 Lys4 tri-methylation (H3K4me3) on *FT* chromatin [20–22]. Indeed, mutants either defective in H3K27me3

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http://dx.doi.org/10.1016/j.plantsci.2013.03.009

or H3K4 demethylation exhibited early-flowering phenotype due to elevated *FT* expression [22,23]. Expression of *FLC*, responding to vernalization-and autonomous pathways was also shown to entail extensive chromatin remodeling [10,24–26]. Interestingly, mutations defective in POLYCOMB REPRESSIVE COMPLEX2 (PRC2)-mediated H3K27me3 such as *curly leaf* (*clf*) or *embryonic flower 2* (*emf2*) caused ectopic expression of not only *FT*, but also *FLC* and *FLC* relatives, negative regulators of flowering [23,27]. Thus, PRC2-mediated control over flowering-related genes has been suggested to function for fine tuning competence for flowering [19].

The PRC2 complex consist of four core proteins; Enhancer of zeste (E(z); an H3K27 methyl transferase), Extra sex comb (Esc), Suppressor of zeste 12 (Su(z)12) and Nurf-p55 in *Drosophila* [28]. In *Arabidopsis*, whereas there exists *FERTILIZATION-INDEPENDENTENDOSPERM* (*FIE*) and *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*), encoding the homologues of ESC and p55, respectively, as a single copy gene, there are three different homologues of E(Z) [CURLY LEAF (CLF), SWINGER (SWN) and MEDEA (MEA)] and of SU(Z)12 [EMF2, VERNALIZATION2 (VRN2) and FERTILIZATION INDEPENDENT SEED 2 (FIS2)] [29]. Depending on the composition of the complex, PRC2 complexes cause tri-methylation at H3K27 on the respective target chromatins, resulting in transcriptionally silenced states in various tissues. PRC2-dependent developmental aspects range throughout plant life cycle, including seed development, endosperm development, leaf development, reproductive phase change and vernalization-dependent flowering [29]. In insects and mammals, the deposited H3K27me3 by PRC2 complex are recognized by PRC1 complex [30,31]. In *Arabidopsis*, a chromodomain protein, LIKE HETEROCHROMATIN1 (LHP1) was shown to partly fulfill the PRC1 function [32]. LHP1-bound chromatins were shown to, largely if not completely, overlap with the H3K27me3 chromatins [20,33]. Despite of extensive progress on the function of PRC2 complex for the past decade, it remains poorly understood how the expression and activity of PRC2 complex are regulated.

Here we report a dominant early-flowering mutant in *Arabidopsis thaliana*, designated as *accelerated flowering 1-1D* (*afl1-1D*). The *afl1-1D* mutant exhibited pleiotropic phenotypes such as curly leaf, semi-dwarfism, and reduced apical dominance. Expression analysis and double mutant studies with late flowering mutants in various floral pathways indicated that elevated *FT* is responsible for the early-flowering of *afl1-1D* mutant. Interestingly, not only flowering-related genes, but also several floral homeotic genes were ectopically overexpressed in the *afl1-1D* mutants in both *FT*-dependent and -independent manner. Chromatin immunoprecipitation analysis implicated that the ectopic up-regulation of several developmental regulatory genes are associated with the reduced degree of H3K27me3 in their chromatins. Together with the results of genetic interaction studies between *afl1-1D* and *lhp1-4*, we propose that *AFL1* defines a novel genetic locus that controls multiple aspects of development through chromatin modification that involves H3K27me3.

2. Materials and methods

2.1. Plant materials and growth conditions

All of the mutants used in this work are in the Columbia (Col) background. The mutants of *lhp1-4* [34], *clf-28* (SALK_139371), *co-1* [5], *gi-2* [35], *ft-1* [5], *ld-1* [36], and *fve-1* [37] has been previously described. The double mutants were generated by genetic crossing. Though *afl1-1D* was isolated in the course of generation of activation-tagging pools using pSKI015 [38], it turned out to be unlinked with basta-resistance, a selection marker of pSKI015. Further physiological and double mutant analysis was performed with homozygous mutant *afl1-1D* lines after back-crossing to wild-type at least three times. Otherwise stated, the plants were grown on half-strength Murashiege-Skoog medium containing 1% sucrose. After growth for 5 d under continuous light condition, the seedlings were transferred to soil for further the vegetative growth under specified photoperiodic conditions at 22–24 °C. For ambient-temperature experiments, the plants were grown under long day condition (16 h L/8 h D) at 16 °C. Flowering time measurement was performed as described [38].

2.2. Construction of double mutant lines

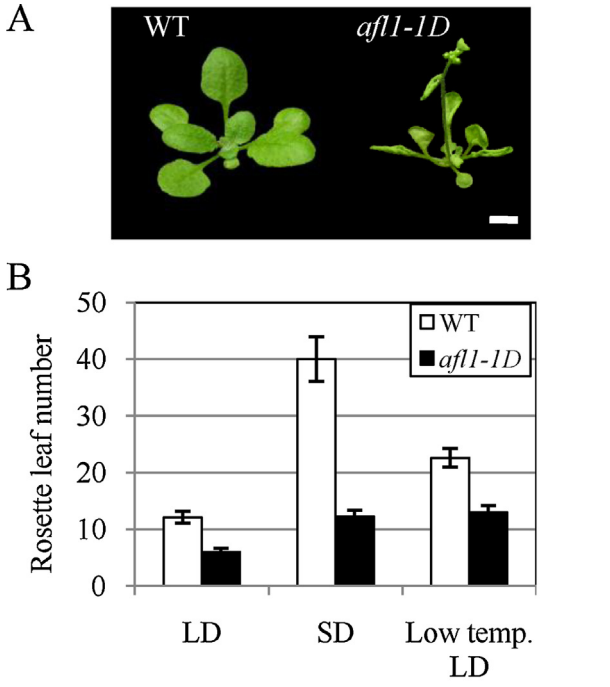
To construct double mutants, we crossed *afl1-1D* mutant plants with a series of late-flowering mutants or *lhp1-4* mutant and allowed the F_1 progeny to self-pollinate to produce the F_2 seeds. Early-flowering plants with curled leaf were identified among the F2 seedlings and then grown for setting F3 seeds. The resulting F3 lines were tested for heterozygous early-flowering/curly leaf and homozygous late-flowering or terminal-flower phenotype (*lhp1-4* mutant phenotype) under long-day conditions. From these, plants with early-flowering/curly leaf phenotypes were selected and further grown to the F4 generation, and then plants were screened for homozygous early-flowering/curly leaf phenotypes. The resulting homozygous lines were designated as double mutants and used for phenotypic analysis.

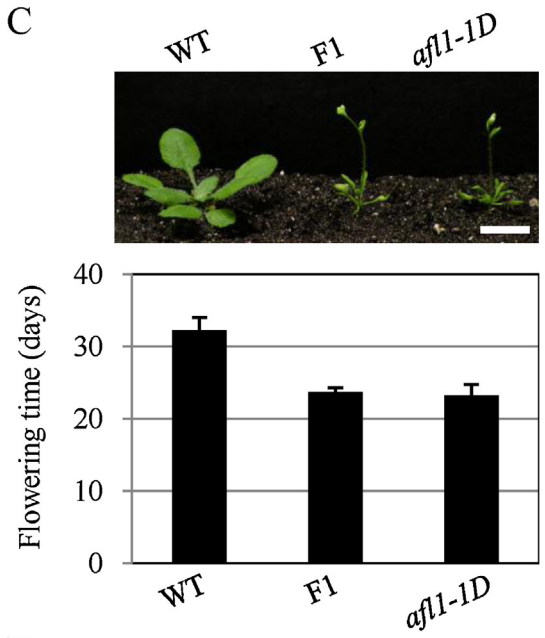
2.3. Gene expression analysis

Total RNA was isolated from 10 d-old seedlings that grown continuous light or short day light (10 h L/14 h D) condition using TRI Reagent (Molecular Research Center, INC.). First strand cDNA was synthesized from 1 μg of RNA after treatment with DNase I using RevertAid™ M-MuLV Reverse Transcriptase (Fermentas). The gene expression was analyzed by semi-quantitative RT PCR analysis or quantitative Real Time-PCR (qRT-PCR). qRT-PCR was performed in 96-well blocks with BioRad iQ-5 real-time PCR system using the EVER Green I master mix (Solis BioDyne) in a volume of 20 μl. The reactions were performed in triplicate for each run. The comparative $_{\triangle\triangle}$CT method was used to evaluate the relative quantities of each amplified product in the samples [39]. The threshold cycle (Ct) was automatically determined for each reaction by the system set with default parameters. Primers used for qRT-PCR have been described [40,41], as listed in Supplementary Table 1.

2.4. Chromatin immunoprecipitation analysis (ChIP)

ChIP analysis was performed essentially as described by Han et al. [42] using 10 d-old plants grown in short day (10 h L/14 h D) light condition. In brief, leaves were vacuum infiltrated with 1% formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking process. Chromatin was isolated and sonicated into 0.5 to 1 kb fragments. Specific antibody against H3K27me3 (Upstate 07-449) was added to the chromatin solution, which had been precleared with salmon sperm DNA/Protein A agarose beads (Upstate 16-157). After subsequent incubation with salmon sperm DNA/Protein A agarose beads, immunocomplexes were precipitated and eluted from the beads. Cross-links were reversed, and residual proteins in the immunocomplexes were removed by incubation with proteinase K, followed by phenol/chloroform extraction. DNA was recovered by ethanol precipitation. The relative amount of immunoprecipitated *FT*, *FLC*, *AG*, *SEP3*, *Ta3*, and *ACTIN1* chromatins were determined by PCR analysis. Primers used have been described [11,22,32,40,41], as listed in Supplementary Table 2.





D

Cross	generation	leaf morphology		χ2	
		leaf rolling	normal		
WT x *afl1-1D*	F1	8	0		
	F2	481	153	0.2	p > 0.5

χ2 was based on the hypothesis of single recessive Mendelian inheritance (3:1)

Fig. 1. Identification of an early-flowering mutant, *afl1-1D*. (A) The early flowering phenotypes of *afl1-1D*. The shown are representative 24 d-old plants under long day condition (16 h L/8 h D). Scale represents 2 mm. WT, wild type. (B) Flowering phenotype of wild type (WT) and *afl1-1D* under various conditions. The flowering time was presented as number of rosette leaves at the time of bolting. The shown are means from at least ten plants. Error bars indicate standard deviations. (C) Dominant phenotype of *afl1-1D*. The plants were grown under long day light condition (16 h L/8 h D). Upper figure showed representative 22 d-old plants. Scale represents 10 mm. For flowering time analysis, at least ten plants were scored to measure average days to flower (lower figure). Error bars indicate standard deviations. WT, wild type. (D) Genetic analysis of *afl1-1D*. The plants scored were grown under long day condition.

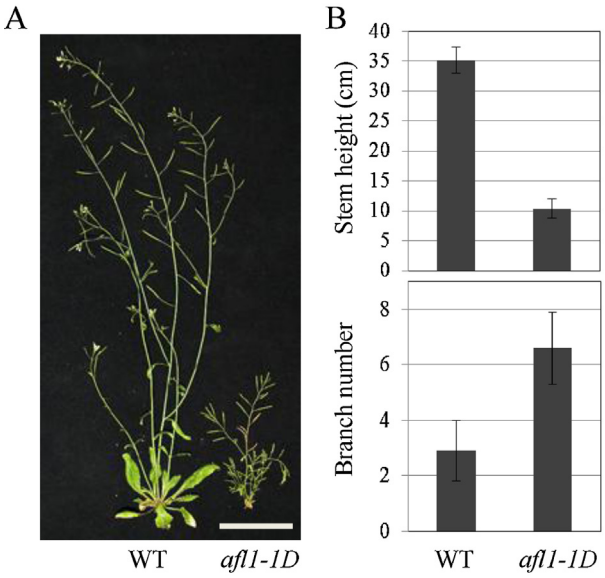


Fig. 2. Pleiotropic phenotypes of *afl1-1D*: dwarfism, curly leaf and reduced apical dominance. (A) Representative phenotype of 45 d-old wild type (WT) and *afl1-1D*. The plants were grown under long day light condition (16 h L/8 h D). Scale represents 5 cm. (B) Average value of stem height and branch number of plants grown as in (A). Error bars indicate standard deviations ($n > 10$).

2.5. Genetic mapping

The chromosomal location of *afl1-1D* mutation was determined by genetic mapping using SSLP (for Simple Sequence Length Polymorphism) markers, as described by Lukowitz et al. [43]. F2 seeds were obtained from the cross between *afl1-1D* mutant (Col background) and Ler plants, and then scored for early flowering/curly leaf phenotypes under long-day condition. Genomic DNA was prepared from wild type-like plants and used for SSLP mapping. For fine mapping of *afl1-1D*, we generated SSLP markers which detect polymorphism between Ler and Col, based on the Insertion/Deletion polymorphism (InDel) data (The Arabidopsis Information Resource, TAIR). The primers used are listed in Supplementary Table 3.

3. Results

3.1. Identification of afl1-1D

We isolated early-flowering mutant designated *accelerated flowering 1-1D* (*afl1-1D*) from activation-tagged mutant pools in *A. thaliana* (Fig. 1A and B). The *afl1-1D* mutant displayed characteristic early flowering and curly leaf phenotype, and those phenotypes were used as marker of genetic analysis of *afl1-1D*. Genetic analysis showed that *afl1-1D* mutant is a single, dominant mutant (Fig. 1C and D). The early-flowering phenotype of *afl1-1D* was not constitutive, exhibiting photoperiod-dependency as well as ambient temperature-sensitivity (Fig. 1B). These results implied that *afl1-1D* is not directly involved in photoperiod- or ambient temperature-dependent flowering pathways. In addition to the early-flowering phenotype, *afl1-1D* exhibited pleiotropic developmental defects including semi-dwarfism, reduced apical dominance, small and curly leaf phenotypes (Figs. 1 and 2). Although *afl1-1D* was isolated from activation tagging pool, the T-DNA insertion were shown to

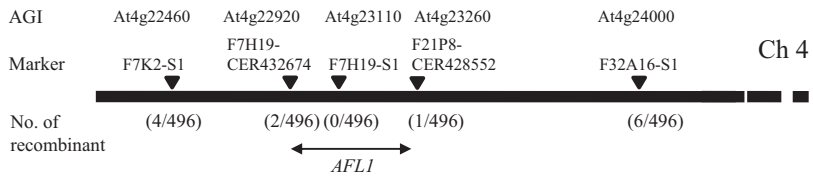


Fig. 3. Chromosomal mapping of *AFL1* locus. The mapping position of the *AFL1*locus is shown along with the actual number of recombinants in parenthesis. The molecular markers tested are depicted as triangles, along with nearby Arabidopsis Genome Initiative (AGI).

be not linked with the phenotypes of *afl1-1D* (data not shown). Thus we determined the chromosomal position of *AFL1* by genetic mapping. Initial mapping results with a set of SSLP markers [43] revealed that *AFL1* resides in the middle of chromosome 4. Further fine mapping was performed by generating molecular markers to narrow down the *AFL1* locus between Arabidopsis Genome Initiative At4g22920 and At4g23260 (Fig. 3). Since there are no known genes to affect flowering within the mapped region, these results imply that *afl1-1D* might define a novel genetic locus in *A. thaliana*. Toward understanding the molecular mechanisms of *afl1-1D* mutant, further physiological and molecular analyses were performed with *afl1-1D* mutant after backcrossing with wild-type four times.

3.2. Expression analysis of flowering-related and homeotic genes in afl1-1D

To further characterize the early flowering phenotype of *afl1-1D* at molecular level, we investigated the expression of flowering-related genes in the *afl1-1D*. While the expression of *CO* and *SOC1* were marginally altered, transcript level of *FT* was significantly elevated in *afl1-1D* mutant, compared to wild type (Fig. 4). Remarkably, the expression of flowering repressor, *FLC* was also increased in *afl1-1D* (Fig. 4). Intriguingly, pleiotropic phenotypes of *afl1-1D* such as the curly leaf phenotype as well as early-flowering are reminiscent of mutants that are defective in PRC2-mediated gene repression including *clf, emf2* or *fie* mutants (Supplementary Fig. S1). Indeed, as reported [23], under the same experimental condition, *clf-28* mutant exhibited similar alterations in the expression level of several floral integrator genes, including up-regulation of both *FT* and *FLC* (Fig. 4). These results led us to hypothesize that *afl1-1D* mutant might affect other target genes of PRC2-mediated repression such as several floral homeotic genes, besides flowering-related genes [44–46]. Thus, we tested whether several homeotic genes, including *AGAMOUS (AG), SEPALLATA3 (SEP3)*, *FRUITFULL (FUL)* and *KNAT1* were overexpressed in *afl1-1D*. The result showed that several homeotic genes including *AG*, *SEP3* and *FUL* but not *KNAT1* were also up-regulated in both *afl1-1D* and *clf*-28 mutants. On the contrary, we could hardly detect any transcript of *Ta3*, a retroelement in heterochromatin, from both wild type and *afl1-1D* mutant (data not shown). These results implicate that *afl1-1D* may affect the expression of various genomic loci at euchromatins, not specified to flowering-related loci.

3.3. FT is epistatic to afl1-1D to promote flowering

To assess the mechanism of early flowering phenotype of *afl1-1D* further, we performed genetic interaction analysis. To this end, we generated double mutants by genetic crossing between *afl1-1D* and a series of late-flowering mutants (Fig. 5A). When combined with *co-1* mutant, that is defective in photoperiodic induction of *FT*, *afl1-1D* could suppress the delayed flowering phenotype of *co-1*, implying that *afl1-1D* might act independently or at downstream of CO. In contrast, a *ft* loss-of-function mutant, *ft-1*, could suppress completely the early-flowering phenotype of *afl1-1D* mutant (Fig. 5B). These double mutant analyses indicated that *FT* is epistatic to *afl1-1D* at least in flowering regulation. We also constructed double mutant lines between *afl1-1D* and late-flowering mutants defective in autonomous pathway, including *fve-1* and *ld-1*. The double mutants exhibited intermediate phenotypes in regard to the flowering time, compared to each parental single mutant (Fig. 5A), suggesting that *afl1-1D* acts independently of autonomous pathways.

While *FT* was shown to be indispensable for the early-flowering phenotype of *afl1-1D*, the small and curly leaf phenotype of *afl1-1D* was not suppressed by *ft-1* mutation (Fig. 5B). Moreover, the increased expression of *SEP3* and *FUL* in *afl1-1D* was partially, not completely, abolished by *ft-1* mutation (Fig. 5C). The results showed that *SEP3* and *FUL* overexpression in *afl1-1D* mutant occurred in both *FT*-dependent and *FT*-independent way.

3.4. Reduced H3K27 trimethylation on various genomic loci in afl1-1D mutant

The remarkable phenotypic similarity between *afl1-1D* and *clf-28*, impaired in PRC2-mediated gene silencing led us to postulate that the pleiotropic effects of *afl1-1D* mutation might be associated with altered chromatin status, particularly H3K27me3,

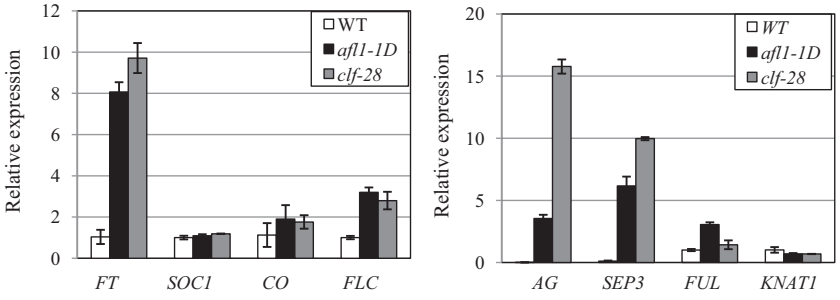


Fig. 4. Expression analysis of selected set of flowering-related genes and homeotic genes. The total RNA was extracted from 10 d-old seedlings grown under continuous light condition. The reverse-transcribed cDNA was subject to quantitative real time-PCR (qRT-PCR) analysis. *PP2A* was used as internal control. The transcript levels of wild type (WT) were set to 1 after normalization by *PP2A*. The shown are average values of experimental triplicates. Error bars represents standard deviations. Similar results were repeated twice with independent biological set.

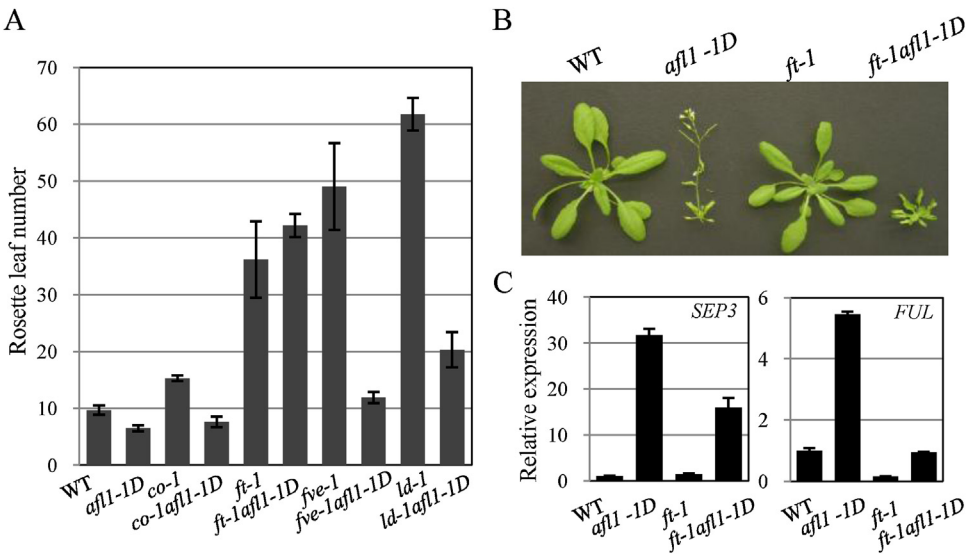


Fig. 5. Double mutant analysis between *afl1-1D* and a set of late-flowering mutants. (A) Flowering time. The flowering time was presented as number of rosette leaves at the time of bolting ($n > 10$). Plants were grown under long day (LD) light condition (16 h L/8 h D). Error bars indicate standard deviations. WT, wild type. (B) Representative phenotypes of *afl1-1D ft-1*double mutant. The picture was taken from 24 d-old plants grown under LD. (C) Expression of *FUL* and *SEP3* in *ft-1afl1-1D*. Total RNA was extracted from 10 d-old seedlings grown under continuous light. Transcript levels of *SEP3* and *FUL* were analyzed by qRT-PCR analysis. *PP2A* was used as internal control. The transcript levels of wild type (WT) were set to 1 after normalization by *PP2A*. The shown are average values of experimental triplicates. Error bars indicate standard deviations. Similar results were repeated twice with independent biological set.

a PRC2-mediated repressive mark. To test the hypothesis, we investigated the level of H3K27me3 on chromatins of *FT*, *FLC*, *AG* and *SEP3*, of which transcript level were up-regulated in *afl1-1D* mutant. Based on the well-characterized genomic region as marked with H3K27me3 in each locus, we performed chromatin immunoprecipitation (ChIP) analysis using antibody against H3K27me3. Compared to wild type, *afl1-1D* mutant exhibited reduced level of H3K27me3 on *FT*, *FLC*, *AG* and *SEP3* (Fig. 6). These results indicated that the ectopic expression at various genomic loci in *afl1-1D* is associated with the decreased level of H3K27me3 of their chromatins. Although H3K27me3 is also associated with repression of heterochromatic retroelements such as *Ta3*, the level of H3K27me3 of *Ta3* was marginally affected by *afl1-1D* (Supplementary Fig. S2). In consistent, we could hardly detect the transcript of *Ta3* in both wild type and *afl1-1D* (data not shown). Thus, these results indicated that *afl1-1D* reduced the repressive mark, H3K27me3 in a subset of euchromatic loci.

3.5. Double mutant analysis of lhp1-4afl1-1D

To further characterize the involvement of *afl1-1D* in H3K27me3-mediated gene regulation, we examined genetic interaction between *afl1-1D* and *LHP1*, a PRC1-like component that mediates H3K27me3-dependent gene repression [20,33,34]. For the purpose, we made *afl1-1Dlhp1-4* double mutant by genetic crossing. Under the same condition, the double mutant flowered as early as its parental single mutant, *afl1-1D* and *lhp1-4* mutant. In agreement to the flowering phenotype, the *FT* expression level of the double mutant was similar to that of its parental single mutants (Fig. 7C), indicating that both *lhp1-4* and *afl1-1D* act in the same pathway in regard to flowering (Fig. 7B and C). It was notable that *afl1-1D* and *lhp1-4* exhibited additive interactions in regard to the stem length and number of siliques, as evidenced by more severe symptoms of *afl1-1Dlhp1-4*, compared to its parental single mutants (Fig. 7A and C). On the other hand, *lhp1-4* and *afl1-1Dlhp1-4* double mutant produced typical terminal-flower morphology, whereas the flowers of *afl1-1D* plants and wild-type did not. Thus, the mode of interactions of *afl1-1D* with *lhp1-4* was shown to be variable, depending on the developmental context.

4. Discussion

Flowering control in higher plants involves multifaceted combinatorial regulation by endogenous as well as external stimuli.

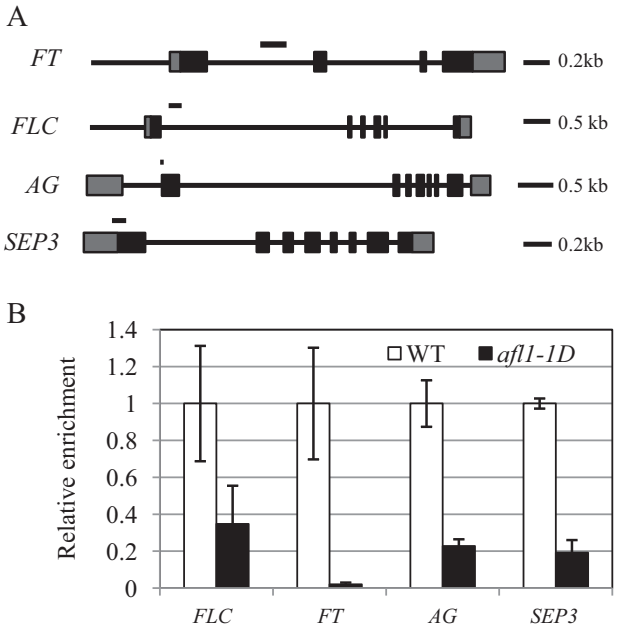


Fig. 6. Decreased trimethylation of H3K27 on *FT*, *FLC*, *AG*, and *SEP3* chromatins by *afl1-1D* mutation. (A) Schematic genome structure of *FT*, *FLC*, *AG* and *SEP3* loci showing regions amplified by primers used for ChIP analysis. The gray boxes indicate 5′ and 3′ UTR respectively. The black boxed indicated exons, while lines indicate introns and intergenic regions. (B) ChIP assay of *FT*, *FLC*, *AG* and *SEP3* chromatin with antibody against H3K27me3. Plants were grown in short-day condition (10 h L/14 h D) for 10 d and harvested for ChIP assay. The degree of H3K27me3 on chromatin was analyzed by real-time PCR. For real-time PCR analysis, the degree of H3K27me3 of chromatin in wild-type (WT) and *afl1-1D* mutant was normalized by 10% input. The data was expressed as relative amount of H3K27me3 in tested region of chromatin when wild-type (WT) levels were set to 1. Error bars indicate standard deviations of experimental triplicates. Similar results were repeated twice with independent biological set.

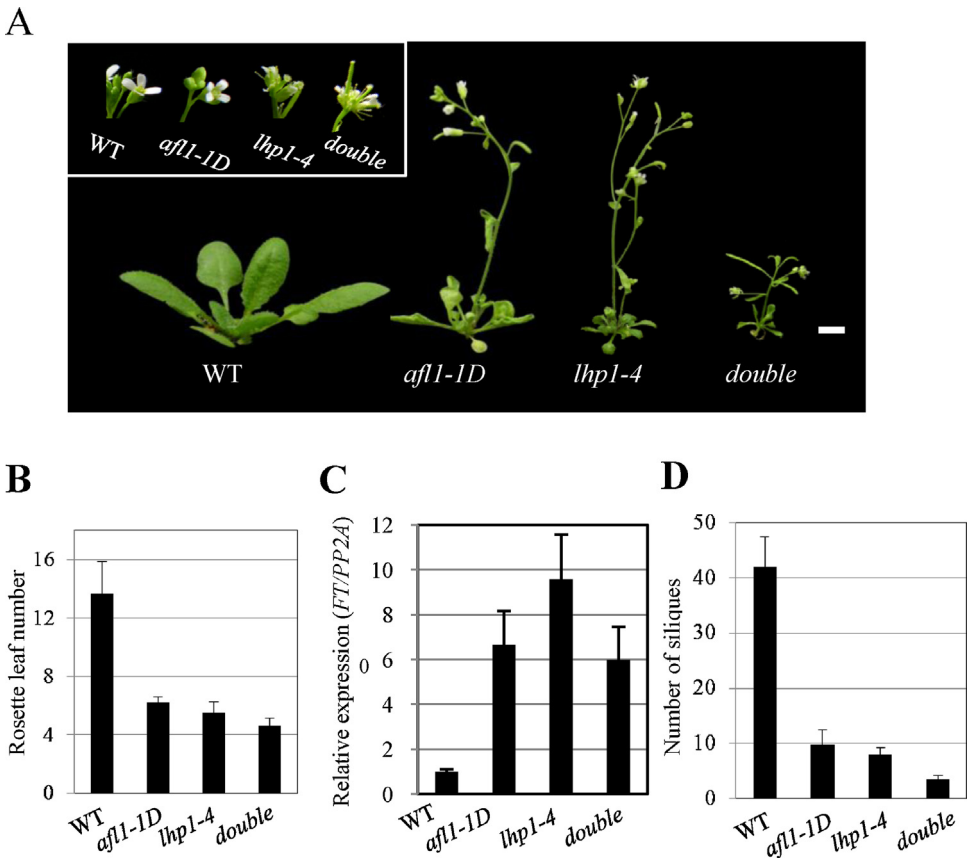


Fig. 7. Double mutant analysis between *afl1-1D* and *lhp1-4*. (A) Representative morphology 27 d-old plants under long-day condition (16 h L/8 h D). Scale represents 5 mm. The magnified view on top of inflorescence of mature plants are shown in inset. WT, wild type. (B) Flowering time of *afl1-1Dlhp1-4*. Average values of rosette leaf number at the time of bolting are shown ($n > 10$). Error bars indicate standard deviations. (C) Expression analysis of *FT*. Total RNA was extracted from 10 d-old seedlings grown under continuous light. The reverse-transcribed cDNA was subject to quantitative real time-PCR (qRT-PCR) analysis. The transcript level of wild type (WT) was set to 1 after normalization by *PP2A*. The shown are average values of experimental triplicates. Error bars indicate standard deviations. (D) Number of siliques in *afl1-1Dlhp1*. Average values of numbers of siliques were obtained from primary inflorescence of 45 d-old plants ($n = 10$). Error bars indicate standard deviations.

Recent elegant studies highlighted the central role of chromatin remodeling in the flowering control that entails various types of chromatin modification on several floral integrator genes [19]. In the present study, we report a novel dominant *Arabidopsis* mutant *afl1-1D* with pleiotropic developmental defects, including early-flowering, curly-leaf, reduced apical dominance and semi-dwarfism (Figs. 1 and 2). Based on our physiological and molecular analysis, we propose that *afl1-1D* impact on various developmental aspects via chromatin modification, associated with H3K27me3. Molecular analysis and double mutant analysis demonstrated that the early-flowering phenotype of *afl1-1D* results from elevated expression of *FT*, presumably due to reduced H3K27me3 on *FT* chromatin (Figs. 4–6). It is worthwhile to note that, despite of elevated expression of *FT*, *afl1-1D* mutant exhibited still photoperiod-sensitive as well as temperature-sensitive flowering phenotypes (Fig. 1), implying *afl1-1D* may not alter photoperiod- or ambient temperature-mediated regulation. In agreement, *afl1-1D* mutant displayed rhythmic diurnal expression of *CO*, *FLC* and *FT* (Supplementary Fig. S3). Thus, *afl1-1D* activated *FT* expression, not affecting its responsiveness to external as well as endogenous stimuli.

Besides *FT*, a flowering repressor, *FLC* as well as several floral homeotic genes such as *SEP3*, *FUL*, and *AG* were found to be ectopically expressed in *afl1-1D* mutant, independently of *FT* (Figs. 4 and 5). Again, we found that level of H3K27me3 of *FT*, *SEP3* and *AG* chromatins was reduced in *afl1-1D* mutant (Fig. 6). The decreased H3K27me3 level at *SEP3* chromatin is in good agreement with the result that ectopic expression of *SEP3* in *afl1-1D* is partly, not completely, dependent on *FT* (Fig. 4), although *SEP3* is one of the target genes of *FT*. These results are in line with recent finding that *SEP3* is under the control of H3K23me3-associated chromatin modification by *CLF*, independently of *FT* [47].

In contrast, there was no discernible difference in the heterochromatic H3K27me3 mark of *Ta3* chromatin between wild type and *afl1-1D* mutant (Supplementary Fig. S2). Consistently, we also could hardly detect the transcript of *Ta3* in both wild type and *afl1-1D* mutant (data not shown). Taken together, these results implicate that *afl1-1D* is involved in H3K27me3-associated chromatin modification of various euchromatic loci, not of heterochromatin.

In *Arabidopsis*, it has been suggested that various PRC2-complexes, consisting of different subunits, mediate trimethylation of H3K27 at various target chromatins, exerting both overlapping and distinct functions throughout developmental stages [29]. In line with the reduced H3K27 level at various genomic loci in *afl1-1D*, the pleiotropic effects of *afl1-1D* resembled those of *clf-28* mutant, deficient in a histone methyltransferase of PRC2-complex (Fig. 4, Supplementary Fig. S1). However, notably, phenotypic severity was variable between *afl1-1D* mutant and *clf-28*, depending on the target genes or developmental context. For example, *afl1-1D* displayed more severe curly-leaf phenotype than *clf-28* mutant (Supplementary Fig. S1), whereas *clf-28* caused more increased level of ectopic *AG* expression, than *afl1-1D* (Fig. 4). Considering functional redundancy as well as diversity among multiple PRC2 complexes, it is plausible that *afl1-1D* may be primarily, if not exclusively, involved in trimethylation of H3K27 that is dependent on CLF-PRC2 complex. The hypothesis of functional involvement of

afl1-1D in the PRC2-mediated gene regulation was further supported by the evidence of genetic epistasis between *afl1-1D* and *lhp1-4* in regard to flowering (Fig. 7). Intriguingly, *afl1-1D* and *lhp1-4* mutants exhibited additive interaction in other developmental aspects than flowering-control, including stem elongation and reproductive organ numbers. The results suggest that *afl1-1D* might be involved in additional processes, independently of *LHP1*. Alternatively, because there exist multiple other plant PRC1-like components such as EMF1, RingA1/A2, other than LHP1 [29], the additive interaction between *afl1-1D* and *lhp1-4* might reflect epistatic relationship between *afl1-1D* and other PRC1-like components. Thus, further analysis on genetic interaction between *afl1-1D* and other components of PRC2-related complex as well as other PRC1-like components would be helpful to clarify the role of *afl1-1D* in H3K27me3-dependent gene repression. On the other hand, it is notable that *afl1-1D* did not affect all of the known PRC2-dependent development. For example, it was rarely found that *afl1-1D* mutant produced abnormal flowers, compared to *clf* and *lhp1* mutants that produce flowers with homeotic alterations (Fig. 7 and unpublished observation). In addition, *afl1-1D* mutant did not cause homeotic defects in leaf development, unlike to *FIE*-silenced mutants [48]. In agreement, the expression of *KNAT1* was shown to be marginally altered by *afl1-1D* (Fig. 4). Thus, it is plausible that *afl1-1D* affect a subset of genes under the control of H3K27me3-mediated gene regulation in a specified developmental context.

How *afl1-1D* mutation impairs H3K27me3 remains unclear. Our genetic mapping of *AFL1* locus revealed that there are no known PRC2-related or flowering-related genes in the mapped region, suggesting that *afl1-1D* defines a novel genetic component (Fig. 3). There are several possibilities how *afl1-1D* impact on H3K27me3-associated chromatin modification. Firstly, *afl1-1D* may repress the expression of certain PRC2-related genes, as exemplified by *pklpkr2* mutation that reduced H3K27me3 level by down-regulation of PRC2 components, including *EMF2*, *CLF*, and *SWN* [49]. However, contrary to expectation, the tested genes encoding components of PRC2 complex were not down-regulated, but rather slightly up-regulated in *afl1-1D* mutant, particularly in the case of *MEA* (Supplementary Fig. S3). Cross-regulation among PRC2-related genes has not been unprecedented. For example, mutants impaired in PRC2-regulated gene regulation such as *emf1* and *fie* mutants were shown to overexpress *MEA* transcript [48]. Nonetheless, it remains to be tested whether *afl1-1D* may down-regulate other components of PRC2 complex. Secondly, *afl1-1D* might hamper the function of a subset of PRC2 complex by overexpression of negative regulator(s) or production of a poisonous protein for functional PRC2 complex. As recent research has begun to unveil novel PRC2 components as well as several types of post-translational regulation of the PRC2 complex [50–56], it will be of interest to examine the functional aspects of PRC2 complex in *afl1-1D* mutant, including stability, chromatin-affinity, and interactome of PRC2 complex. Thirdly, the reduced H3K27me3 in *afl1-1D* may be due to increased activity of counter-acting chromatin remodeller including trithorax-group proteins [57]. Moreover, as chromatin remodeling often exhibits interdependency or cross-regulation among chromatin modification processes such as histone H2b monoubiquitination, histone acetylation, H3K4me3, H3K9me2, H3K36me2 and DNA methylation [58–60], thus, it is conceivable that *afl1-1D* might impact on directly other types of chromatin modification, resulting in reduction of H3K23me3 level. Finally, it cannot be also excluded that *afl1-1D* might be involved in mitotically stable maintenance of H3K27me3 mark after establishment. Recently, a cellular machinery involving ICU2, a DNA polymerase alpha subunit, was shown to be necessary for maintenance of mitotically stable H3K27me3 at both euchromatin loci and heterochromatic loci [40,61]. Unlike to *icu2* mutant, *afl1-1D* did not affect the stable repression of *Ta3*, a heterochromatic retroelement (Supplementary Fig. S2 and data not shown). Therefore, it is less likely that *afl1-1D* affect the components for maintenance itself. Although we found that several genes in the mapped region were up-regulated in *afl1-1D* mutant, it does not necessarily indicate that one of the overexpressed genes is the corresponding *AFL1* gene, because *afl1-1D* may affect expression of those loci, presumably via control of H3K27me3. Indeed, unfortunately, so far, we failed to recapitulate the *afl1-1D* mutant phenotypes by transgenic overexpression of those overexpressed genes. Our ongoing several approaches to identify the molecular nature of *AFL1* gene, including genome sequencing, suppressor analysis and recapitulation with the overexpressed genes would provide critical information about the function of *afl1-1D*. Despite of the lack of information on the molecular nature of *afl1-1D*, nonetheless, we believe that our study provide useful information as well as genetic material for plant biology community, particularly interested in epigenetic developmental regulation.

In summary, our present genetic identification and physiological characterization of *afl1-1D* mutant provides a useful genetic resource for further genetic and biochemical dissection on the regulatory roles of chromatin modification, particularly associated with H3K23me3, during development of *A. thaliana.*

Acknowledgements

We thank Dr. Detlef Weigel for providing activation-tagging vector, pSKI015. We also thank the ABRC for providing mutant seeds. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-C00049, KRF-2007-C00706), Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0070016, 2009-0027318), the Next-Generation Biogreen 21 program (Plant Molecular Breeding Center No. PJ008137 and SSAC PJ008180) by the Rural Development Administration.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.03.009.

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