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Growth habit determination by the balance of histone methylation activities in *Arabidopsis*

Jong-Hyun Ko[1,2], Irina Mitina[1,2], Yosuke Tamada[3,5], Youbong Hyun[1], Yeonhee Choi[1], Richard M Amasino[2,3], Bosl Noh[2,4,*] and Yoo-Sun Noh[1,2,*]

[1]School of Biological Sciences, Seoul National University, Seoul, Korea, [2]Global Research Laboratory for Floral Regulatory Signaling, Seoul National University, Seoul, Korea, [3]Department of Biochemistry, University of Wisconsin, Madison, WI, USA and [4]Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju, Korea

In *Arabidopsis*, the rapid-flowering summer-annual versus the vernalization-requiring winter-annual growth habit is determined by natural variation in *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*). However, the biochemical basis of how FRI confers a winter-annual habit remains elusive. Here, we show that FRI elevates *FLC* expression by enhancement of histone methyltransferase (HMT) activity. EARLY FLOWERING IN SHORT DAYS (EFS), which is essential for *FRI* function, is demonstrated to be a novel dual substrate (histone H3 lysine 4 (H3K4) and H3K36)-specific HMT. FRI is recruited into *FLC* chromatin through EFS and in turn enhances EFS activity and engages additional HMTs. At *FLC*, the HMT activity of EFS is balanced by the H3K4/H3K36- and H3K4-specific histone demethylase (HDM) activities of autonomous-pathway components, RELATIVE OF EARLY FLOWERING 6 and FLOWERING LOCUS D, respectively. Loss of HDM activity in summer annuals results in dominant HMT activity, leading to conversion to a winter-annual habit in the absence of FRI. Thus, our study provides a model of how growth habit is determined through the balance of the H3K4/H3K36-specific HMT and HDM activities.

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Introduction

Plants have evolved various growth habits, such as annual, biennial, and perennial, as evolutionary adaptations to specific climates and regions. *Arabidopsis* accessions can be either vernalization-requiring winter annuals or rapidly flowering summer annuals (Michaels *et al*, 2003). The winter-annual habit is conferred by the activation of the major floral repressor *FLOWERING LOCUS C* (*FLC*) by *FRIGIDA* (*FRI*; Koornneef *et al*, 1994; Lee *et al*, 1994; Johanson *et al*, 2000). Mutations in autonomous-pathway *FLC* repressors also result in winter-annual behaviour in the absence of functional *FRI* (Koornneef *et al*, 1991; Baurle and Dean, 2006). Although a number of genetic factors affecting *Arabidopsis* growth habits have been identified (Baurle and Dean, 2006), the biochemical function of the natural winter-annual determinant, FRI, and the molecular mechanism by which autonomous-pathway mutations delay flowering and convert a summer annual to a winter annual are not well understood.

EARLY FLOWERING IN SHORT DAYS (*EFS* or Su(var), E(z), and Trithorax (*SET*) *DOMAIN GROUP 8*) is essential for the winter-annual behaviour of lines with functional *FRI* (Kim *et al*, 2005; Zhao *et al*, 2005) and encodes a SET domain-containing protein grouped with *Drosophila* ASH1 (Springer *et al*, 2003), a controversial multi-catalytic histone methyltransferase (HMT; Beisel *et al*, 2002; Li *et al*, 2007; Tanaka *et al*, 2007). Although EFS is essential for FRI activity, its function in *FLC* regulation has been studied primarily in the absence of *FRI*. In fact, *fri efs* plants were reported to have reduced levels of histone H3 lysine 36 di- and tri-methylation (H3K36me2/me3) in the *FLC*-coding region (Zhao *et al*, 2005; Xu *et al*, 2008).

Histone methylation, which affects chromatin structure and gene transcription, is balanced by the activities of HMTs and histone demethylases (HDMs; Klose and Zhang, 2007). Here, we describe, at a molecular level, how the summer- versus winter-annual habit of *Arabidopsis* is determined by balanced activities of HMTs and HDMs acting at *FLC*. After being recruited by EFS, FRI allows for HMT activities to dominate at *FLC*. In the process, EFS and RELATIVE OF EARLY FLOWERING 6 (REF6; Noh *et al*, 2004) exert mutually antagonistic functions as novel dual substrate (H3K4 and H3K36)-specific HMT and HDM, respectively.

Results and Discussion

EFS is crucial for FRI recruitment into FLC chromatin and both EFS and FRI are required for elevated H3K4me/H3K36me

We evaluated the effect of EFS and FRI at the *FLC* locus by chromatin immunoprecipitation (ChIP) assays. Both FRI and EFS were required for the elevated levels of H3K4me3 and H3K36me2 (Figure 1A and B), markers related to transcriptional activation and elongation, respectively (Li *et al*, 2007). FRI- and EFS-mediated elevation of H3K4me3 was observed throughout the *FLC* regions evaluated, whereas H3K36me2 levels were elevated within the internal gene body. Partially functional FRI:β-glucuronidase (FRI:GUS; Supplementary

*Corresponding authors. B Noh, Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Korea. Tel.: +82 2 871 6675; Fax: +82 2 871 6673;
E-mail: bnoh2003@yahoo.co.kr or YS Noh, School of Biological Sciences, Seoul National University, Shillim-Dong, Gwanak-Gu, Seoul 151-747, Korea. Tel.: +82 2 880 6674; Fax: +82 2 871 6673;
E-mail: ysnoh@snu.ac.kr
[5]Present address: National Institute for Basic Biology, Okazaki, Aichi 444-8585, Japan

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Figure S1) and almost fully functional EFS:FLAG (Supplementary Figure S2) fusion proteins associated directly with the 5′ region and both the 5′ and internal regions of *FLC*, respectively (Figure 1C and D).

Surprisingly, the association of FRI:GUS with *FLC* chromatin was abolished in the absence of EFS or SUPPRESSOR OF FRIGIDA 4 (SUF4; Figure 1C), a FRI-interacting nuclear protein (Kim *et al*, 2006). However, the association of EFS:FLAG with the 5′ region of *FLC* was not significantly affected by FRI, whereas its interaction with the gene body was reduced by the presence of FRI (Figure 1D). Co-immunoprecipitation (Co-IP) of FRI:GUS and EFS:FLAG (Figure 1E) suggested that FRI and EFS might be present in the same complex, although the data did not exclude the possibility of interaction between the FLAG-tagged EFS and GUS instead of FRI. Consistent with a possible *in vivo* interaction, the C-terminal region of EFS interacted with a functional FRI ($FRI^{Sf\text{-}2}$) and SUF4 but not with a non-functional FRI (FRI^{Col}) in yeast two-hybrid assays (Figure 1F). Together, these results demonstrate that EFS is crucial for the recruitment of FRI to *FLC* chromatin, as well as for the elevation of H3K4me3 and H3K36me2 levels at *FLC*.

FRI was reported to be required for the maintenance of *FLC* expression in late embryos, but dispensable for the initial reactivation in early embryos during reprogramming (Choi *et al*, 2009). Like FRI, EFS was also required for the maintenance, but not for the initial reactivation of *FLC* (Supplementary Figure S3). Therefore, FRI and EFS act at similar developmental stages.

EFS is a novel dual substrate (H3K4/H3K36)-specific HMT performing the functions of both SET1 and SET2 of yeast

In yeast and animals, H3K4 and H3K36 methylations are catalysed by SET1/Trithorax-class HMTs and SET2-class HMTs, respectively (Hampsey and Reinberg, 2003; Shilatifard, 2008). Arabidopsis Trithorax1 (ATX1) together with its homolog ATX2 and ATX-related 7 has a function in elevating H3K4me3 at *FLC* (Pien *et al*, 2008; Tamada *et al*, 2009). FRI was shown to be required for the recruitment of WDR5a into *FLC* chromatin, and WDR5a in turn interacts with ATX1 (Jiang *et al*, 2009). Hence, EFS-dependent elevation of H3K4me3 at *FLC* in lines with *FRI* might be catalysed either by ATX1/ATX2 recruited through WDR5a/FRI/EFS or by EFS directly.

Therefore, it was of interest to examine the residue-specific HMT activity of EFS. Although full-length EFS could not be obtained, we could express the SET domain of EFS (EFS-SET), which contains the catalytic core elements (SET and cysteine-rich (C) pre-SET or post-SET domains; Figure 1G), as a GST fusion protein (Figure 1H). In assays using calf thymus histones as substrates followed by immunoblot analyses with various methylation-specific antibodies (abs), EFS-SET increased the levels of H3K4me2/me3 and H3K36me2/me3 but not of H3K4me1, H3K36me1, H3K9me3, and H3K27me3 (Figure 1I). When oligonucleosomes were used as substrates, the levels of H3K4me3 and H3K36me2 were also increased by EFS-SET, and the extent of H3K36me2 increased when FRI was added to the reaction (Figure 1I). Thus, EFS appears to possess intrinsic H3K4 and H3K36 di- and tri-methyltransferase activities that, at least in part, account for the FRI/EFS-mediated increased H3K4me3 and H3K36me2 levels within *FLC* chromatin (Figure 1B). The FRI-independent effects of EFS on H3K4 and H3K36 methylations at *FLC* are shown in Figure 3. In summary, EFS might have two functions in *FLC* activation: first, as a scaffold recruiting the FRI-containing transcriptional co-activator complex, and second, as a novel HMT with dual substrate (H3K4me1/me2 and H3K36me1/me2) specificity, thus performing both SET1 and SET2 functions.

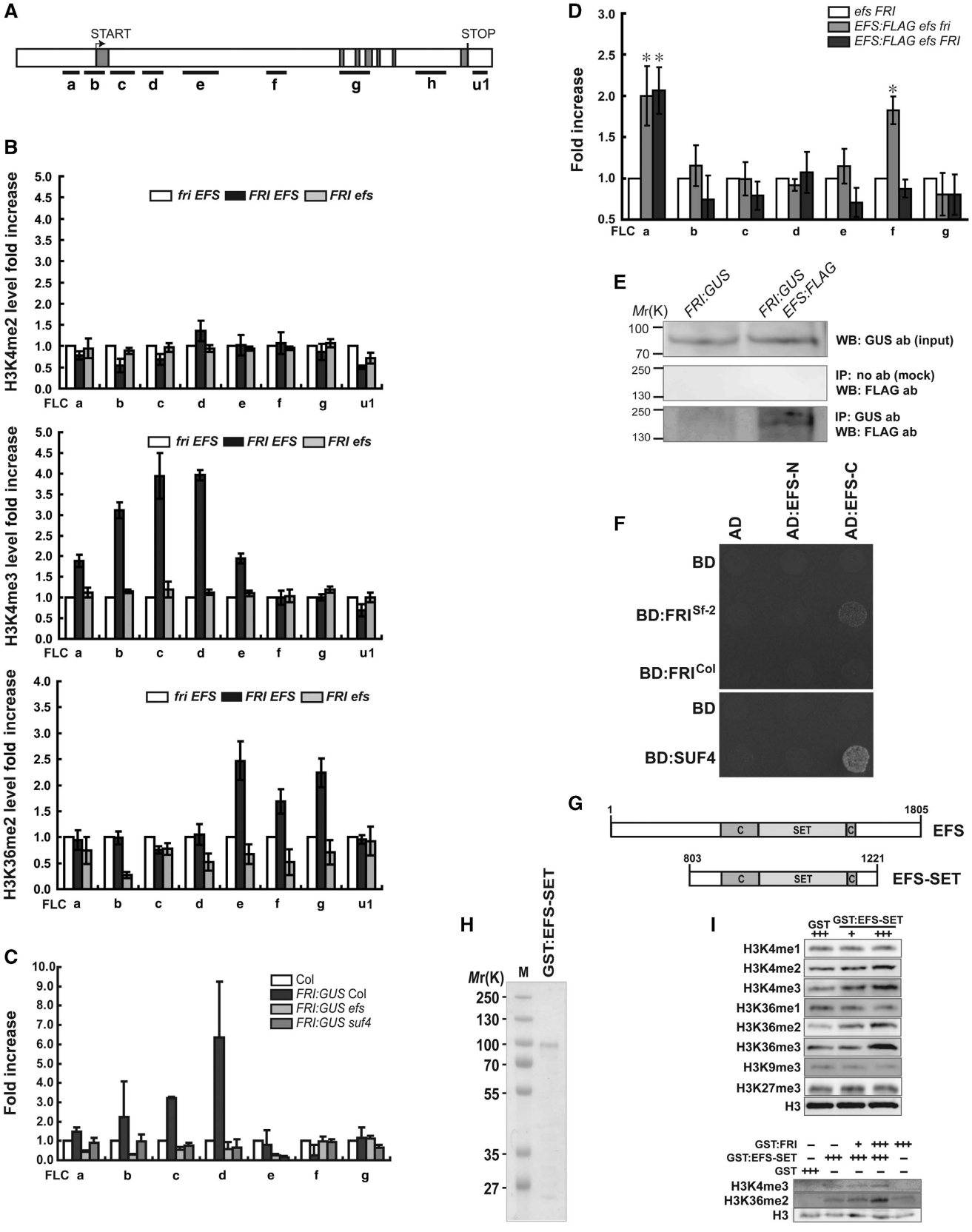
REF6 is a novel dual substrate (H3K4/H3K36)-specific HDM repressing FLC chromatin together with FLD

Similar to the effects of functional FRI, the loss of autonomous-pathway members with non-functional *fri* also results in *FLC* activation and a winter-annual habit. REF6 (Noh *et al*, 2004) and FLOWERING LOCUS D (FLD; He *et al*, 2003) are two autonomous-pathway members predicted to have HDM activities based on the presence of the Jumonji C (JmjC) domain in REF6 and on the sequence similarity of FLD to human LSD1-class amine oxidases (AOs), although their biochemical functions have not yet been demonstrated. FCA and FPA, two other autonomous-pathway members, were also reported to be involved in chromatin silencing through an RNA-mediated pathway (Baurle *et al*, 2007), and FCA appears to require FLD for *FLC* repression (Liu *et al*, 2007).

The JmjC domain of REF6 is most related to the JmjC domains of human JARID1 family members, which are H3K4me2/me3-specific demethylases (Christensen *et al*, 2007). AOs such as FLD are capable of demethylating H3K4me1/me2 (Shi *et al*, 2004). Consistent with the predicted functions of REF6 and FLD, H3K4me2/me3 levels in the 5′ region of *FLC* were increased in *ref6* and *fld* mutants in comparison to wild type (Figure 2A). In addition, *ref6* but not *fld* mutants also contained increased H3K36me2 levels in the internal gene body (Figure 2A). Although the lack of demethylase activity of AOs for tri-methylated lysines (Shi *et al*, 2004) makes it hard to explain the increased H3K4me3 levels in *fld*, these results suggest that REF6 and FLD might have additive functions in *FLC* repression as H3K4/H3K36- and H3K4-specific HDMs, respectively. The *ref6 fld* double mutants had higher *FLC* mRNA levels (Figure 2B and C) and showed a more delayed flowering (Figure 2D) than either of the single mutants, also supporting their independent repressive functions. Direct association of the functional REF6:GUS (Noh *et al*, 2004) with *FLC* chromatin was observed in the internal gene body as well as in the 5′ region, whereas the interaction of the functional GST:FLD (Supplementary Figure S4) only occurred in the 5′ region (Figure 2E and F). Thus, the two proteins bound to *FLC* chromatin in regions where histone methylation levels were also significantly increased in the corresponding mutants. The elevated level of *FLC* mRNA but not H3K36me2 at the *FLC* locus in *fld* mutants suggests that the deposition of H3K36me2 can be uncoupled from transcriptional activity.

As no JmjC proteins have been demonstrated to demethylate both H3K4me and H3K36me, we tested the intrinsic HDM activity of REF6 by using 6xhistidine-tagged full-length REF6 (REF6:6xHis; Figure 2G). REF6:6xHis, but not ΔREF6:6xHis, which lacks the JmjN and JmjC domains of REF6, strongly reduced the levels of H3K4me2 and H3K36me2 in the substrate calf thymus histones and also moderately reduced the

levels of H3K4me3 and H3K36me3 (Figure 2G). H3K4me1, H3K36me1, H3K9me3, and H3K27me3 levels were not affected by REF6:6xHis in the assays. Together with the increased levels of H3K4me2/me3 and H3K36me2 at *FLC* in *ref6* mutants (Figure 2A) and the direct binding of REF6:GUS with *FLC* chromatin (Figure 2E), these results indicate that REF6 is an H3K4me2/me3- and H3K36me2/me3-specific HDM.



The HMT activity of EFS is antagonized by the HDM activities of REF6/FLD in FLC regulation

The results shown in Figures 1 and 2 suggest potential antagonism between EFS and REF6 for H3K4me and H3K36me and between EFS and FLD for H3K4me at *FLC*. To test these possibilities, we first measured H3K4me3 levels at *FLC* using *ref6 efs* and *fld efs* double mutants. The increased H3K4me3 caused by the *ref6* or *fld* mutation was suppressed in the double mutants to the levels found in the *efs* single mutant (Figure 3A). Similarly, the *ref6* mutation-induced increase of H3K36me2 was also suppressed by *efs* (Figure 3B). Changes of the histone markers were correlated with the steady-state mRNA levels of *FLC* (Figure 3C and D) and flowering time (Figure 3E and F). The *efs* mutation efficiently suppressed *ref6*- or *fld*-induced increases of *FLC* mRNA expression and delayed flowering. The minor residual expression of *FLC* in *fld efs* (Figure 3D) implies an EFS-independent portion of FLD activity. However, the results in Figure 3 indicate that the FRI-independent H3K4/H3K36 methyltransferase activity of EFS is largely responsible for the de-repression of *FLC* in *ref6* and *fld* and that EFS has antagonistic biochemical functions to REF6 or FLD in H3K4me/H3K36me or H3K4me, respectively, at the *FLC* locus.

The summer- versus winter-annual habit of Arabidopsis is determined by the balance of H3K4/H3K36-specific HMT and HDM activities

On the basis of our results, we propose a model for the summer- versus winter-annual habit in *Arabidopsis* through balanced histone methylation activities (Figure 4). In non-functional *fri*-containing summer annuals, the HDM activities of REF6 and FLD are dominant to the HMT activity of EFS because of lack of FRI, resulting in low H3K4me/H3K36me and *FLC* mRNA levels. However, in mutants of the HDMs or certain other autonomous-pathway members, the HMT activity of EFS overrides the decreased HDM activities, and as a result, the H3K4me/H3K36me and *FLC* mRNA levels are increased, leading to winter-annual behaviour. In *FRI*-containing winter-annual types, EFS recruits FRI as a scaffold, and in turn, FRI enhances the HMT activity of EFS and recruits additional H3K4-specific HMTs (ATXs). Thus, the bifurcate enhancement of HMT activities driven by the EFS/FRI complex causes a winter-annual habit.

The mechanism of the FRI enhancement of EFS activity remains unknown. *FRI* and its relatives form a small, plant-specific gene family (Michaels *et al*, 2004). In the crucifer lineage, FRI appears to have been recruited for *FLC* activation (Johanson *et al*, 2000). It will be of future interest to explore whether other FRI family members act in higher plants as plant-specific co-activators for H3K4 and H3K36 methyltransferases, or other histone-modifying complexes, at a range of target loci.

Materials and methods

Plant materials, growth conditions, and flowering time analyses

All Arabidopsis mutants used in this study are in the Columbia (Col) background and were described previously: *FRI* (Lee *et al*, 1994), *ref6-3* (Noh *et al*, 2004), *fld-3* (He *et al*, 2003), *efs-3* (Kim *et al*, 2005), and *suf4* (Kim *et al*, 2006). Plants were grown and their flowering times were measured as described (Han *et al*, 2007). At least 15 plants for each genotype were scored for the flowering time analyses, and data are shown with means ± s.d. Photoperiods used in the study were 16 h light and 8 h dark for LD and 8 h light and 16 h dark for SD.

FRI:GUS, EFS:FLAG, REF6:GUS, and 35S::GST:FLD

The *FRI:GUS* (Choi *et al*, 2009) and *REF6:GUS* (Noh *et al*, 2004) constructs were described previously. The *EFS* fragment including the native promoter region was amplified by PCR from genomic DNA and cloned into pEarleyGate302. The *FLD* cDNA was cloned into pGEX4T and the *GST*-fused *FLD* fragment was PCR amplified and cloned behind the *CaMV35S* promoter in Cameleon-YC2.1, resulting in the binary *35S::GST:FLD* construct. Binary plasmids were introduced into *Agrobacterium tumefaciens*, and the resulting strains were used to transform *Arabidopsis* plants using the floral dip method (Clough and Bent, 1998). Sequences of primers used for constructions are available on request.

ChIP assay

Fourteen-day-old seedlings grown in LD were used for the ChIP experiments as previously described (Han *et al*, 2007). ChIP DNA was analysed by qPCR as described in the RT–PCR and qPCR section using *FLC* genomic primers FLC a to h (Liu *et al*, 2007) and U1 (Sung and Amasino, 2004). 'Input' is 10% of the nuclear extract used and 'mock' refers to the control lacking ab. Abs used are described in the '*In vitro* HDM assay' section. For the ChIP assays using *FRI:GUS* and *EFS:FLAG*, GUS ab (Invitrogen A5790) and FLAG ab (Sigma A8592) were used, respectively. Each ChIP assay was repeated at least three times with independent samples. The values are the means of these biological replicates and error bars represent s.e.

Histochemical GUS imaging

The *FLC:GUS* transgene in *FRI EFS* was introduced into *fri EFS* and *FRI efs* by crossing. The *GUS* activity in the gametophytes and seeds was analysed as described previously (Choi *et al*, 2009).

Co-IP

For the Co-IP assay, we generated the double transgenic *EFS:FLAG*- and *FRI:GUS*-containing homozygous plants by crossing. Cross-linked nuclear proteins were extracted from LD-grown 14-day-old seedlings according to the ChIP protocol. After dilution of the nuclear proteins with immunoprecipitation buffer (50 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM DTT, 1 mM PMSF), FRI:GUS was immunoprecipitated with GUS ab (Invitrogen A5790). The FRI:GUS-containing immunocomplex was resolved by SDS–PAGE and detected by FLAG ab (Sigma A8592).

GST:EFS-SET and GST:FRI protein expression

The cDNA sequences encoding the pre-SET, SET, and post-SET domains (amino acids 803–1221) of EFS or functional FRI were amplified by RT–PCR and subcloned into the *Sal*I and either *Bam*HI

Figure 1 FRI- and EFS-dependent H3K4 and H3K36 methylation of *FLC* chromatin. (**A**) *FLC* locus with exons (grey boxes) and introns (white boxes) showing regions tested for ChIP. (**B**) ChIP-quantitative real-time PCR (qPCR) analyses of *FLC* chromatin with indicated histone antibodies (abs). *fri EFS* is wild-type Columbia (Col). Error bars represent s.e. (**B**–**D**). (**C**, **D**) ChIP-qPCR to test the direct association of FRI:GUS (**C**) or EFS:FLAG (**D**) with *FLC* chromatin using GUS (**C**) or FLAG (**D**) ab. (*$P<0.05$ versus control in a Student's *t*-test). (**E**) Co-IP of FRI:GUS and EFS:FLAG. Western blot (WB) was performed with the indicated abs. (**F**) Interaction of EFS with FRI and SUF4 in yeast two-hybrid assays. The N-terminal (EFS-N) or C-terminal region (EFS-C) of EFS were fused to the GAL4 activation domain (AD). FRI from Sf-2 or Col and SUF4 were fused to the GAL4-binding domain (BD). (**G**) Domains in EFS and EFS-SET. (**H**) Coomassie-stained purified GST:EFS-SET. (**I**) HMT activity of GST:EFS-SET in assays using calf thymus histones (upper) or oligonucleosomes (lower) as substrates.

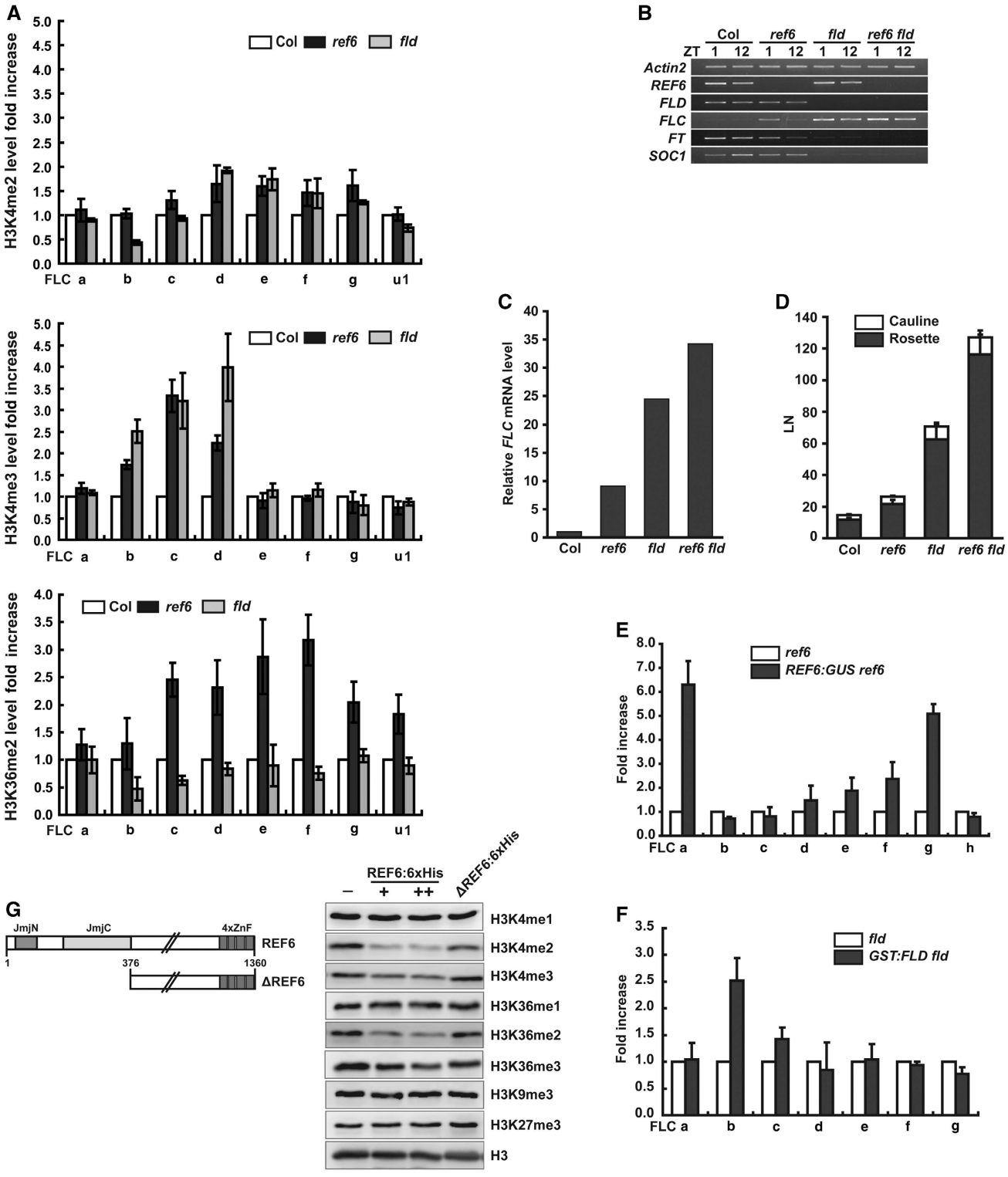


Figure 2 Repression of *FLC* chromatin by REF6 and FLD. (**A**) ChIP–qPCR analyses of *FLC* chromatin for regions described in Figure 1A. Error bars represent s.e. (**B**, **C**) Expression of flowering genes at zeitgeber (ZT) 1 and 12 in *ref6*, *fld*, and *ref6 fld* as studied by reverse transcription (RT)–PCR (**B**) or RT–qPCR at ZT2 (**C**). *FT*: *FLOWERING LOCUS T*. *SOC1*: *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*. The values are the means of three technical replicates (**C**). (**D**) Flowering time of *ref6*, *fld*, and *ref6 fld* in LD as scored by the leaf number (LN). Error bars represent s.d. (**E**, **F**) ChIP–qPCR was used to test the direct association of REF6:GUS (**E**) or GST:FLD (**F**) with *FLC* chromatin using GUS (**E**) or GST (**F**) ab. Error bars represent s.e. (**G**) Domains in REF6 and ΔREF6 (left), and HDM activity of REF6:6xHis (right).

or *Eco*RI sites of the pGEX-4T vector (GE Healthcare). Sequences of primers used for construction are available on request. Recombinant GST, GST:EFS-SET, and GST:FRI fusion proteins were expressed in *Escherichia coli* strain BL21 and resuspended in 20 ml of extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.4% Triton X-100, 100 μM PMSF, 1 mg/ml lysozyme), followed by sonication. Cleared soluble proteins obtained after centrifugation were purified using glutathione-Sepharose beads (GE Healthcare) according to the manufacturer's instructions.

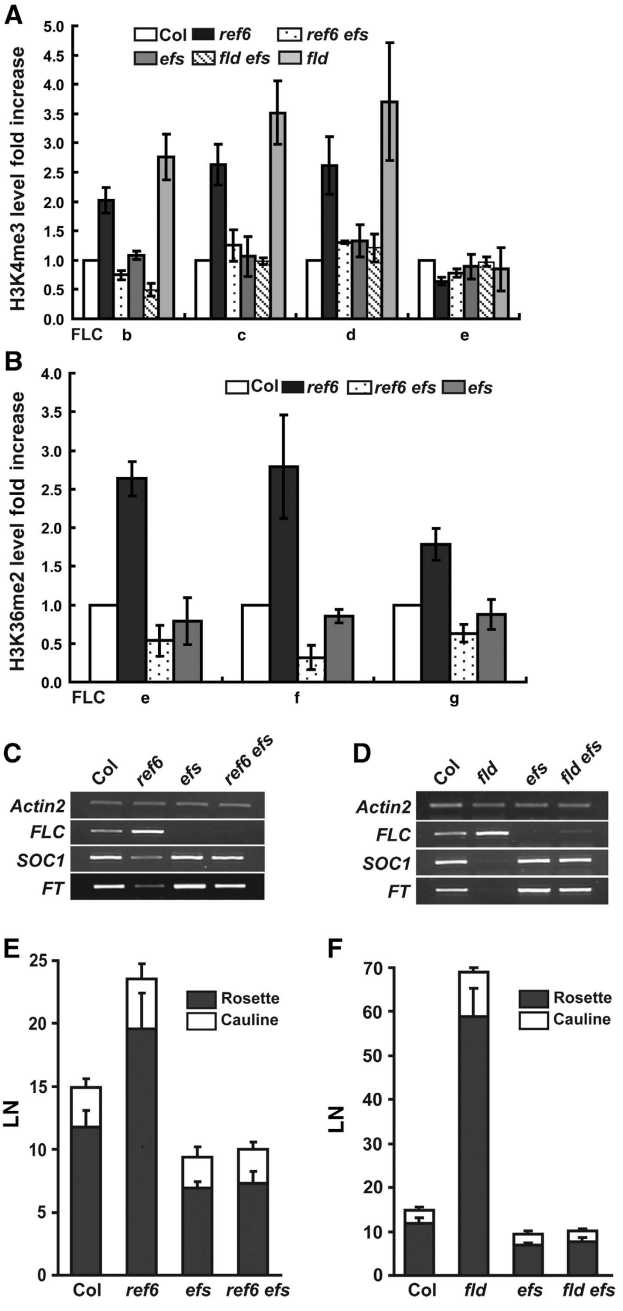


Figure 3 HMT activity of EFS is antagonized by HDM activities of REF6/FLD in *FLC* regulation. (**A**) ChIP-qPCR analyses of *FLC* chromatin with H3K4me3 ab. Regions tested are as described in Figure 1A and error bars represent s.e. (**A**, **B**). (**B**) ChIP-qPCR analyses of *FLC* chromatin with H3K36me2 ab. (**C**, **D**) Expression of flowering genes in *ref6 efs* (**C**) and *fld efs* (**D**) as analysed by RT–PCR. (**E**, **F**) Flowering time of *ref6 efs* (**E**) and *fld efs* (**F**) in LD. Error bars represent s.d.

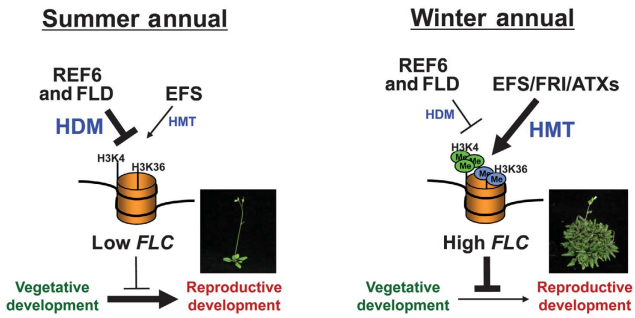


Figure 4 H3K4me- and H3K36me-mediated determination of summer- or winter-annual habit in *Arabidopsis*. See text for details.

In vitro HMT assay

Following a previously published protocol (Rea *et al*, 2000) with modifications, the *in vitro* HMT assay was carried out with 5 μg of calf thymus histones (Sigma) or 1 μg of oligonucleosomes (12-nucleosome array reconstituted with *Xenopus* histones on the G4E4 fragment) as substrates and 0.2 μM of *S*-adenosyl-L-methionine (Sigma) as the methyl donor in 100 μl of methyltransferase buffer (50 mM Tris pH 8.0, 20 mM KCl, 10 mM MgCl2, 10 mM β-mercaptoethanol, 250 mM sucrose). We routinely used 8 μg (+) or 24 μg (+++) of purified GST fusion proteins for the assays. After 13 h incubation at 30°C, reactions were stopped by boiling in SDS loading buffer, the proteins were separated by 15% SDS–PAGE and analysed by western blot with methylation-specific abs, which are described in the '*In vitro* HDM assay' section.

RT–PCR and qPCR

Total RNA was extracted from 14-day-old seedlings grown in LD using the TRI Reagent (Sigma) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the SuperScript First-Strand Synthesis System (Invitrogen) with an oligo(dT) primer. RT–PCR was performed using gene-specific primers as previously described (Song *et al*, 2009).

qPCR was performed with the Applied Biosystems 7300 Real-time PCR System using iQ™ SYBR Green Supermix (Bio-Rad). Absolute quantification was performed by generating standard curves using serial dilutions of *Actin2* and *FLC* sequence-containing clones. The relative mRNA levels represent the fold change over the control. The values are the means of three technical replicates and error bars represent s.d. To compare the relative amounts of the amplified products for ChIP experiments, genomic fragments of the *FLC* locus were amplified by qPCR and calculated according to the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), similar to the controls. *Actin2* with primers JP1565 and JP1596 (Johnson *et al*, 2002) was used as an internal control and for normalization between samples. Control levels were set to 1 after normalization and others were expressed as relative values to the control levels.

REF6:6xHis protein expression

For REF6 expression in yeast, a PCR-amplified full-length *REF6* cDNA fragment was cloned first into the pENTR/SD/D-TOPO entry vector, and then transferred into the pYES-DEST52 destination vector using the Gateway cloning technology (Invitrogen). The resulting plasmid was transformed into yeast strain BY4741 (*MATa*, *ura3Δ0*, *leu2Δ0*, *his3Δ1*, *met15Δ0*; Open Biosystems). The Δ*REF6* mutant clone was created by amplifying a *REF6* cDNA fragment lacking the coding regions for the JmjN and JmjC domains and by cloning into the same entry and destination vectors with the full-length *REF6*. Sequences of primers used for constructions are available on request.

REF6:6xHis and ΔREF6:6xHis proteins were prepared as follows: overnight cultured yeast cells grown in a synthetic SD medium with essential amino acids (Clontech) and 2% glucose were inoculated into 3 l of CSM-URA 2% (w/v) Raffinose and further grown at 30°C to an optical density at 600 nm (OD600) of 0.8. For protein induction, 2% (w/v) peptone, 1% (w/v) yeast extract, and 2% (w/v) galactose were added and further incubated at 30°C to an OD600 of 4–5. Yeast cells were collected by centrifugation and the pellets were suspended in 150 ml of lysis buffer (50 mM Tris pH 7.9, 400 mM NaCl, 1 mM PMSF). Acid-washed glass beads (1 g/10 ml buffer) were added, and the mixture was transferred into a bead beater (Biospec). The suspension was subjected to bursts for 30 s followed by 90 s cooling periods on ice for 2 h. The lysate was clarified by centrifugation and subjected to further purification steps according to the manufacturer's instructions for His-tagged proteins (Qiagen). Purified recombinant REF6 proteins were dialyzed against Hepes buffer (40 mM Hepes pH 7.9, 50 mM KCl, 10% glycerol, 1 mM DTT, 0.2 mM PMSF) overnight at 4°C. On the next day, the dialyzed proteins were concentrated approximately five-fold by ultrafiltration in Microcon centrifugal filters with

a 30-kDa molecular cutoff (Millipore) and stored at −20°C until further use.

In vitro HDM assay

In vitro HDM assays were performed as previously described (Whetstine *et al*, 2006). Briefly, 10 μg (+) or 20 μg (++) of purified REF6:6xHis or 20 μg of purified ΔREF6:6xHis was incubated with 40 μg of calf thymus histones type II-A (Sigma) in the demethylation reaction buffer (20 mM Tris–HCl pH 7.3, 150 mM NaCl, 50 μM $(NH_4)_2Fe(SO_4)_2$ $6(H_2O)$, 1 mM α-ketoglutarate, 2 mM ascorbic acid) for 5 h at 37°C. Histone modifications were detected by western blot with the following abs: H3K4me1 (Upstate 07-436), H3K4me2 (Upstate 07-030), H3K4me3 (Abcam ab8580), H3K36me1 (Abcam ab9048), H3K36me2 (Upstate 07-369), H3K36me3 (Abcam ab9050), H3K9me3 (Upstate 07-442), H3K27me3 (Upstate 07-449), and H3 (Abcam Ab1791-100).

Yeast two-hybrid assay

Vectors and yeast strains were obtained from Clontech (Matchmaker GAL4 Two-Hybrid System 3). Assays were carried out according to the manufacturer's instructions. The cDNA sequences encoding FRI^{Sf-2}, FRI^{Col}, EFS-N (amino acids 1–1218) and EFS-C (amino acids 1210–1805) were amplified by RT–PCR. FRI^{Sf-2} and *EFS-N* were subcloned into *Bam*HI and *Eco*RI sites of pGBKT7 and pGADT7, respectively. EFS-C was subcloned into *Bam*HI sites of pGADT7. FRI^{Col} was subcloned into *Sma*I sites of pGBKT7. Sequences of primers used for construction are available on request. The *SUF4* construct was previously described (Kim *et al*, 2006). Plasmids were co-transformed into yeast AH109 strain by lithium acetate method, and transformants were incubated on the synthetic dropout media lacking Trp and Leu for 4 days at 30°C. Then, cells were transferred to the synthetic dropout media lacking Trp, Leu, His, and adenine and incubated at 30°C until colonies appeared.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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