Planta (2011) 234:1237–1250
DOI 10.1007/s00425-011-1484-y

ORIGINAL ARTICLE

Identification of regulators required for the reactivation of *FLOWERING LOCUS C* during *Arabidopsis* reproduction

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Received: 9 June 2011 / Accepted: 5 July 2011 / Published online: 20 July 2011

Abstract *FLOWERING LOCUS C* (*FLC*) is a central floral repressor for the determination of flowering time in *Arabidopsis*. *FLC* expression is reactivated upon fertilization and regulated during seed development to ensure the appropriate floral behavior; however, the molecular mechanism for this process is largely unknown. Here, we report the identification of crucial regulators for *FLC* reactivation during embryogenesis by analyzing *FLC::GUS* and endogenous *FLC* expression. We newly define that the full reactivation of *FLC* requires a FRIGIDA (FRI)-containing protein complex throughout embryogenesis. Mutations in *EARLY FLOWERING 7* (*ELF7*) and *VERNALIZATION INDEPENDENCE4* (*VIP4*) showed severe defects in the reactivation of *FLC* transcription, suggesting that both of the genes, *Arabidopsis* homologs of the members of the yeast RNA polymerase II-associated factor 1 (Paf1) complex, are indispensable for *FLC* reactivation. *actin-related protein 6* (*arp6*), *arabidopsis trithorax 1* (*atx1*), *arabidopsis trithorax-related 7* (*atxr7*), and *atx1 atxr7* double mutants also caused the downregulation of *FLC* during seed development, but the defects were less severe than those in mutants for the FRI- and Paf1-complexes. These results suggest that the ARP6-containing Swr1-complex and *FLC*-specific histone methyltransferases, ATX1 and ATXR7, have relatively partial roles in *FLC* reactivation. In contrast to the roles of the histone modifiers, factors in the DNA methylation pathway and biogenesis of small RNAs are not involved in *FLC* regulation during reproduction. Taken together, our results demonstrate that adjustment by select *FLC* activators is critical for the re-establishment of an *FLC* expression state after fertilization to ensure competence for optimal flowering in the next generation.

Keywords *FLOWERING LOCUS C* · Epigenetic resetting · Reproduction · *FLC* regulators

Abbreviations

Paf1	RNA polymerase II-associated factor 1
H3K4me3	Trimethylation at histone H3 Lysine4
DAG	Days after germination
qRT-PCR	Quatitative RT-PCR
siRNA	Short interfering RNA
miRNA	Micro RNA
RdDM	RNA dependent DNA Methylation

Introduction

For plants, the timing of the initiation of flowering has been critical during evolution to maximize reproductive success (Amasino 2010). *FLOWERING LOCUS C* (*FLC*), which encodes a MADS box-containing transcription factor, is a central floral repressor in *Arabidopsis* (Michaels and Amasino 1999). High levels of *FLC* expression cause late flowering even under inductive long-day conditions,

Electronic supplementary material The online version of this article (doi:10.1007/s00425-011-1484-y) contains supplementary material, which is available to authorized users.

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whereas transcriptional repression of *FLC* promotes the initiation of flowering (Ausin et al. 2004; Lim et al. 2004; Sheldon et al. 2000). *FLC* and its upstream activator, *FRIGIDA* (*FRI*), are the major determinants of the natural variation of flowering time in *Arabidopsis*: winter annual *Arabidopsis* plants contain dominant alleles of *FLC* and *FRI*. FRI is a plant-specific protein that is required for high levels of *FLC* expression (Johanson et al. 2000; Michaels and Amasino 1999, 2001). In contrast, rapid-cycling summer annuals contain mutations in either *FRI* or *FLC*, and, therefore, *FLC* expression remains very low (Kim et al. 2009; Shindo et al. 2005; Werner et al. 2005). *FRI* is a member of a small gene family, and two other members are *FRI-LIKE 1* and *2* (*FRL1* and *FRL2*), which are required for high levels of *FLC* expression. SUPPRESSOR OF FRIGIDA 4 (SUF4), a C2H2-type zinc-finger protein, has been identified as an FRI-interacting protein (Kim et al. 2006). SUF4 binds directly to the *FLC* promoter and may recruit a protein complex containing FRI to activate *FLC* expression.

Genetic studies have identified various factors required for a high level of *FLC* expression (Fig. 1). Most of them, if not all, are components of chromatin-modifying complexes that promote active chromatin (Amasino 2010; Amasino and Michaels 2010). Mutations in genes that encode homologs of the members of yeast RNA

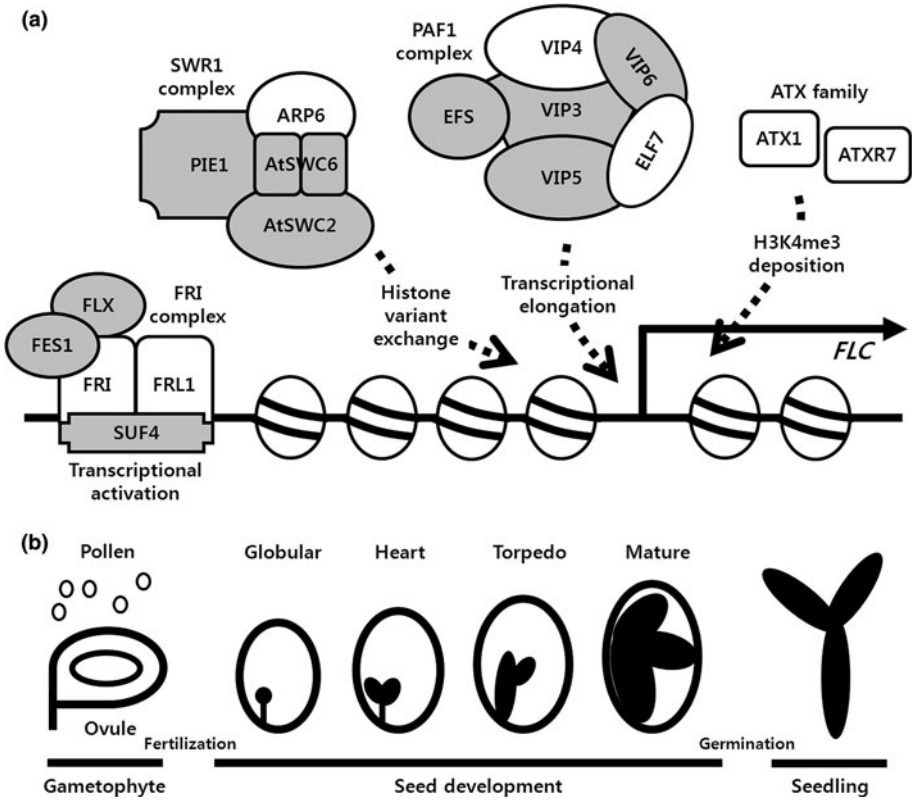

Fig. 1 Schematic diagram for the regulatory pathways of *FLC* activation. **a** Four major genetic pathways are known to be involved in *FLC* activation. FRI-complex binds to the *FLC* promoter and mediates transcriptional activation. FRI, FRL1, SUF4, FES1, and FLX have been isolated as components of FRI-complex. Loss-of-function mutations of *FRI* and *FRL1* cause significant decrease of *FLC* transcription in vegetative tissues. FRI-complex is also known to recruit Swr1-complex to *FLC* region. Swr1-complex is involved in incorporation of histone variant H2A.Z in various organisms and loss-of-biochemical function of Swr1-complex results in the partial reduction of *FLC* expression in vegetative tissues. PIE1, ARP6, AtSWC2, and AtSWC6 have been isolated as components of Swr1-complex in *Arabidopsis*. EFS, ELF7, VIP3, VIP4, VIP5, and VIP6 comprise the Paf1-complex in *Arabidopsis*. Paf1-complex associates with transcriptional machineries and maintains the elongation of transcriptional process by deposition of H3K4 methylation. Loss-of-Paf1-complex function induces the significant reduction of *FLC* expression in vegetative tissues. ATX1 and ATXR7 have been isolated as *Arabidopsis* homolog of TRITHORAX family that mediates H3K4 methylation. Both ATX1 and ATXR7 function redundantly and *atx1 atxr7* double mutant shows partial reduction of *FLC* in vegetative tissues. Protein components examined in this study are marked with white circles or white rounded boxes within each protein complex. **b** Simple diagram of *Arabidopsis* reproduction. *Arabidopsis* produces female gametophyte within the ovule and male gametophyte, pollen, for reproduction. After double fertilization, embryo and endosperm are developed. Based on morphology of embryo, seed development was divided into globular, heart, torpedo, and mature stages in this study. Most of the *FLC* regulatory mechanisms have been studied in seedling stage after germination. By contrast, our study has focused on the roles of various *FLC* activators during reproduction

polymerase II-associated factor 1 (Paf1) complex have resulted in a failure of elevated *FLC* expression, even in the FRI-containing winter annuals. EARLY FLOWERING 7 (ELF7, also known as VERNALIZATION INDEPENDENCE 2 [VIP2]), ELF8 (VIP6), VIP4, and VIP5 are members of the Paf1-complex, which has been shown to be required for a high level of *FLC* expression (He et al. 2004; Kim et al. 2005; Oh et al. 2004) (Fig. 1). In yeast, the Paf1-complex has been found to interact with SET1 and SET2 histone methyltransferases (Belotserkovskaya and Reinberg 2004), and the recruitment of SET1 and SET2 to chromatin has resulted in an increase of methylation in histone H3 lysine 4 and lysine 36 (H3K4 and H3K36), respectively. In *Arabidopsis*, ARABIDOPSIS TRITHORAX (ATX) 1 through 5, and ARABIDOPSIS TRITHORAX-RELATED (ATXR) 1 through 4, and 7 have a SET domain that is homologous to yeast SET1 and *Drosophila* Trithorax (Baumbusch et al. 2001). Mutations in *ATX1* have been shown to cause decreased *FLC* expression with reduced tri-methylation of H3K4 (H3K4me3) at the 5′-end of *FLC* (Pien et al. 2008). In addition, it has been reported that the *atx1 atxr7* double mutant showed additive phenotypes in contrast to those of each of the single mutants, and both ATX1 and ATXR7 histone methyltransferase were required for the enrichment of H3K4me3 in *FLC* chromatin (Amasino 2010) (Fig. 1). EARLY FLOWERING IN SHORT DAYS (EFS, also known as SET DOMAIN GROUP 8 [SDG8]) belongs to the SET2 class that is responsible for H3K36me2 in the promoter region and the first intron of *FLC* (Xu and Shen 2008; Zhao et al. 2005).

In *Arabidopsis*, PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1) (Noh and Amasino 2003), ACTIN-RELATED PROTEIN 6/ SUPPRESSOR OF FRIGIDA 3/EARLY IN SHORT DAYS 1 (ARP6/ SUF3/ ESD1) (Choi et al. 2005; Deal et al. 2005; Martin-Trillo et al. 2006), and AtSWC6/SERRATED LEAVES AND EARLY FLOWERING (SEF) (Choi et al. 2007; March-Diaz et al. 2007) have been isolated as homologs of members of the yeast Swr1 complex (Fig. 1). These *Arabidopsis* members are involved in the exchange of histone H2A to H2A.Z, and it has been reported these are required for full *FLC* expression (Choi et al. 2007).

Vernalization, a prolonged exposure to cold, establishes the repressive epigenetic histone marker H3K27me3 at *FLC* chromatin. This silencing of *FLC* expression is stably maintained mitotically, even under warm conditions (Gendall et al. 2001; Sung and Amasino 2004), but this "memory of winter" is not transmitted to the next generation and has to be reset during reproduction for the re-establishment of vernalization. Many studies have focused on identifying the *FLC* regulators and their regulatory mechanisms during the vegetative stage, but *FLC* resetting and its mechanisms during the reproductive stage remain largely unknown.

Recently, Sheldon et al. and our group have reported that, regardless of the transcriptional activity in the adult plants, *FLC::GUS* expression was not detected in male or female gametophytes, but the reactivation of both parental alleles occurred after fertilization in the embryo, not in the endosperm (Choi et al. 2009; Sheldon et al. 2008). We also have examined the possibility that these *FLC* regulators are involved in the *FLC* resetting mechanism.

Here, we describe the defects in the reactivation of *FLC* in various mutants for *FLC* regulators that are required for elevating *FLC* expression during the vegetative stage. Our results suggest that some specific activators of *FLC*, rather than canonical mechanisms, play important roles in the reactivation of *FLC* during embryogenesis.

Materials and methods

Plant material and growth conditions

Most of the plants used in our experiment have an active *FRI* allele from SF-2 (Lee et al. 1994) by a genetic cross. In this article, we called Col-0 having an active *FRI* allele the wild type. *fri* refers to the inactive allele of *FRI* from Col-0. All of the plants used in this study are in the Col-0 background, except for *met1-6* in the Col-*gl*, and, *cmt3-7* and *ago4-1* in L*er*, and *drm1 drm2* in the Ws background. Plants were grown under previously described conditions (Choi et al. 2009).

Histochemical GUS imaging

The *FLC::GUS* reference line (Michaels et al. 2005) used in this paper was a gift from S. Michaels and various mutant plants containing *FLC::GUS* transgene were generated by genetic crosses between mutant plants and *FLC::GUS* plant. Methods for *GUS* staining, fixing tissue, and microscopy are as described previously (Choi et al. 2009).

Analysis of gene expression

Total RNA extraction from developing seeds was performed using seeds independently harvested according to the stage of embryo development. The Qiagen RNeasy Plant Mini Kit (Qiagen) was used for RNA extraction from seeds. Genomic DNA was eliminated on-column with a Qiagen RNase-free DNase set. A total of 0.5–1 μg of RNA was reverse transcribed using M-MLV reverse transcriptase (Ambion) and an oligo-dT primer. PCR amplification was performed using gene-specific primers: *TUB2*, 5′-ATCGATTCCGTTCTCGATGT-3′ and 5′-ATCCAGTTCCTCCTCCCAAC-3′; *FLC*, 5′-GAAGAGAACCAGGTTTTGGCTA-3′ and 5′-TTTGTCCAGCAGGTGACATC-3′.

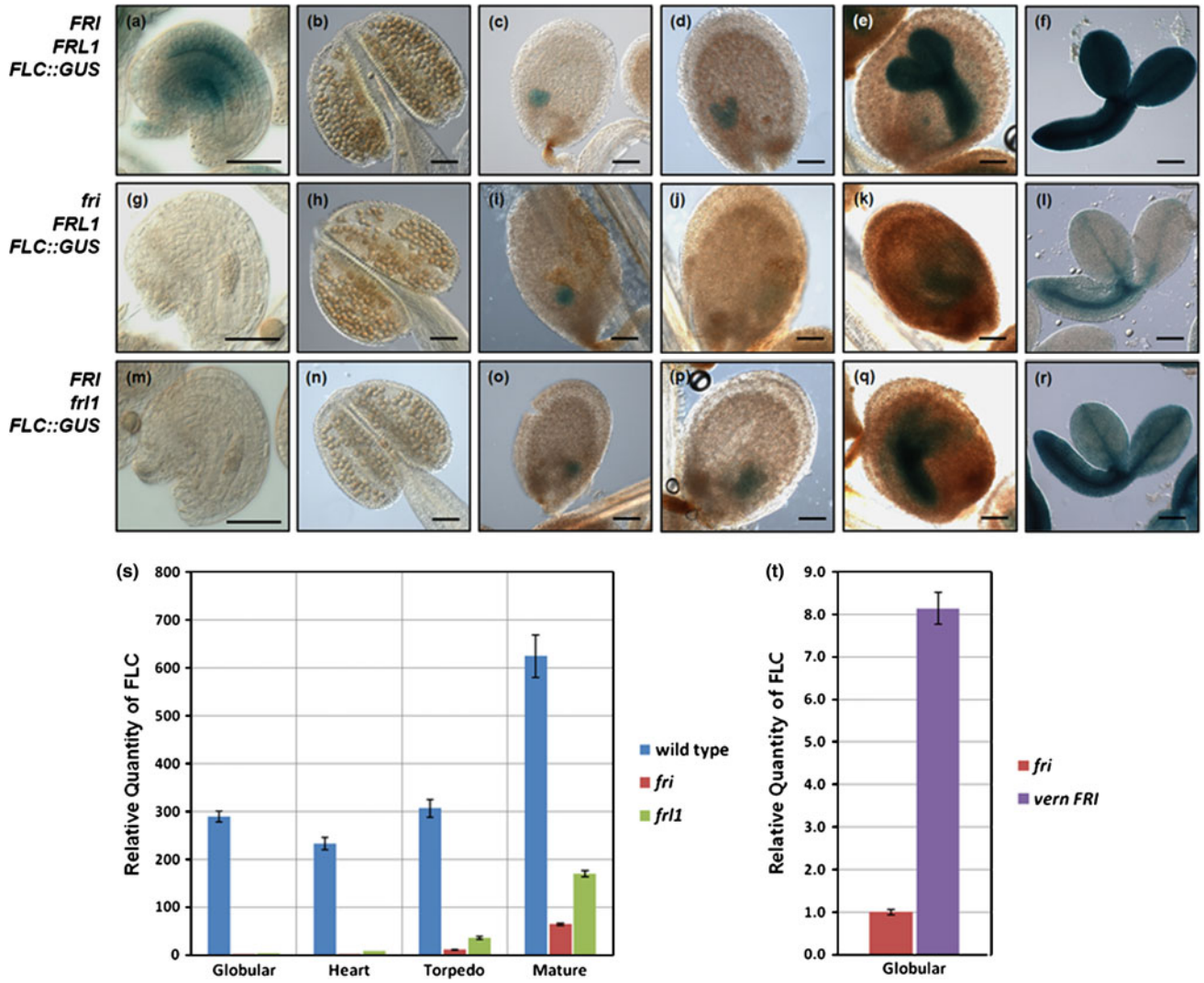

Fig. 2 *FLC::GUS* and *FLC* mRNA expression in *fri* family mutants. *FLC::GUS* expression in the wild type ovule (**a**), stamen (**b**), developing embryos at the globular stage (**c**), heart stage (**d**), torpedo to walking stick stage (**e**) and mature stage (**f**). *FLC::GUS* expression in the *fri* mutant ovule (**g**), stamen (**h**), developing embryos at the globular stage (**i**), heart stage (**j**), torpedo to walking stick stage (**k**) and mature stage (**l**). *FLC::GUS* expression in the *frl1* mutant ovule (**m**), stamen (**n**), developing embryos at the globular stage (**o**), heart stage (**p**), torpedo to walking stick stage (**q**) and mature stage (**r**). **s** Real time qRT-PCR analysis of *FLC* mRNA expression in the developing seeds of wild type, *fri* and *frl1* mutants. **t** Comparison of *FLC* mRNA expression by qRT-PCR in the *fri* mutant with vernalization-treated wild type *FRI* plants in seeds at globular stage. Values in (**s**) and (**t**) are normalized to an internal reference *TUBULIN-β-CHAIN2 (TUB2)* gene and plotted relative to the expression of *FLC* in the *fri* globular seeds, which was set to 1.0. Value represents the average of triplicate measurements ± standard error. Scale bars: ovules, 50 μm; stamens, 100 μm; seeds, 100 μm

Real-time qPCR

Real-time qPCR was performed as described previously (Choi et al. 2009). Reactions for all of the templates were performed in duplicate or triplicate. The threshold cycle (Ct) was determined using the CFX Manager Software (Bio-Rad). Gene-specific transcripts were quantified using the ddCt method ($ddCt = Ct_{gene\ of\ interest} - Ct_{TUB2}$) (Livak and Schmittgen 2001). Real-time SYBR-green dissociation curves showed one species of amplicon for each primer combination.

Results

Effect of the *FRI* family on high levels of *FLC* reactivation during embryogenesis

FLC expression is known to require not only *FRI* but also other *FRI* family members to be competent for vernalization (Michaels et al. 2004; Schlappi 2006) (Fig. 1). To explore the function of the *FRI* family in *FLC* reactivation, we compared the expression pattern of *FLC::GUS* in *fri* and in *frl1* mutants to the expression in the wild type during

the reproductive stage. In the *FLC::GUS* construct (Michaels et al. 2005), *GUS* was inserted into the sixth exon of a 16-kb genomic clone containing 5.4-kb upstream of the *FLC* start site and 5-kb downstream of the stop codon. We introduced the *FLC::GUS* transgene into *fri* or *frl1* mutants by genetic crosses and analyzed the *GUS* expression pattern in the self-fertilized F2 progenies.

In the wild type, *FLC::GUS* activity was not detected in the female or male gametophyte, except in the sporophytic tissues of the ovule (Fig. 2a, b), which are of diploid maternal origin (Choi et al. 2009). After fertilization, *FLC::GUS* was expressed only in embryos, and a high level of *GUS* expression was maintained throughout embryogenesis (Fig. 2c–f). In *fri* or *frl1* mutant plants, *FLC::GUS* was not expressed in either of the gametophytes, as in wild type (Fig. 2g, h, m, n), suggesting that FRI and FRL1 do not function in the regulation of *FLC* expression during gametogenesis. The sporophytic expression of *GUS* in the tissues surrounding the embryo sac disappeared (Fig. 2g, m), indicating a decrease of *FLC* expression in the vegetative tissues of the mutants. After fertilization, *GUS* was detected in the young embryos of both *fri* and *frl1* mutants (Fig. 2i, o). As the embryo matured, however, *GUS* activity gradually decreased from the cotyledons of the embryo (Fig. 2j, k, p, q), and, eventually, *GUS* expression was largely restricted to the vascular regions of both mutants (Fig. 2l, r).

We also performed real-time quantitative RT-PCR (qRT-PCR) to examine the effect of the *FRI* family on the endogenous *FLC* expression. Seeds at the globular stage show higher *FLC* expression than the heart stage, but the *FLC* expression gradually increased as the seed matured (Fig. 2s, blue bar). In *fri* or *frl1* mutants, the overall level of *FLC* mRNA was significantly reduced in both of the mutants (Fig. 2s, red and green bar, respectively). According to our previous report, the sporophytic *GUS* expression of the ovules correlated with the vegetative expression of *FLC*, and it retained a considerable amount of expression in the maternal tissues of globular seeds in the wild type (Choi et al. 2009). In contrast, vegetative *FLC* expression has been reported to be reduced in *fri* and *frl1* (Michaels et al. 2004), and this likely led to the lack of sporophytic *GUS* expression in the ovules of both mutants (Fig. 2g, m). Through vernalization, the vegetative *FLC* expression in a *FRI* plant has been shown to be reduced to a level similar to that in a *fri* plant, even in the presence of *FRI*-complex genes (Michaels et al. 2004). Therefore, we directly compared the level of *FLC* mRNA in *fri* plants to that in vernalized *FRI* plants with seeds at the globular stage. The *FLC* expression of vernalized *FRI* plants was approximately eightfold higher than that of *fri* plants (Fig. 2t). This clearly demonstrates that the reactivation of *FLC* was impaired in *fri*-complex mutants from the early stages of seed development. Taken together, our results suggest that the FRI and FRL1 are essential for the full activation of *FLC* during embryogenesis.

Effects of Swr1- and Paf1-complexes on *FLC* reactivation during embryogenesis

ARP6 encodes nuclear ACTIN-RELATED PROTEIN 6, a homolog of a component of the yeast ATP-dependent chromatin remodeling Swrl-complex (Choi et al. 2005) (Fig. 1). *VIP4* and *ELF7* encode plant relatives of yeast LEO1 and PAF1, respectively, which are components of the Paf1-complex (He et al. 2004; Zhang and van Nocker 2002) (Fig. 1); mutations in these genes have been shown to not only induce early flowering but also result in pleiotropic phenotypes (Dennis and Peacock 2007). To investigate the potential roles of ARP6, VIP4, and ELF7 on *FLC* reactivation, the *FLC::GUS* transgene was introduced into *arp6*, *vip4*, and *elf7* mutants by genetic crosses, and the histochemical *GUS* staining patterns were analyzed in the F2 progenies. Similar to the *fri* and *frl1* mutant plants, *FLC::GUS* expression was detected in neither ovule sporophytic tissues (Fig. 3e, i, m) nor gametophytes (Fig. 3e, f, i, j, m, n). The lack of *GUS* signal in the female and male gametophyte indicates that ARP6, VIP4, and ELF7 do not function in *FLC* expression in gamete cells.

After fertilization, *FLC::GUS* expression in the *arp6* mutant was detected from young embryos, but showed at a reduced level in the mature embryo (Fig. 3g, h) than that in the wild-type mature embryo (Fig 3c, d). We previously observed that *FLC::GUS* activity was significantly reduced in *pie1* mutant (Choi et al. 2009). Both PIE1 and ARP6 are components of the same Swr1-complex (Fig. 1), and the mutations in *pie1* and *arp6* resulted in the reduction of *FLC::GUS* expression.

In contrast, *FLC::GUS* activity was not detected in any of the developmental stages of *vip4* and *elf7* mutant embryos (Fig. 3k, l, o, p), except for a slight expression in the shoot apical meristem of mature embryos in each mutant (arrows in Fig. 3l, p). This result indicates that VIP4 and ELF7 have critical roles in the reactivation, as well as the maintenance, of *FLC* expression during embryogenesis and further suggests the important function of histone modification on *FLC* chromatin by the *Arabidopsis* Paf1-complex during reproduction. In addition, *vip4* and *elf7* mutants also showed an occasional ectopic *GUS* expression in the endosperm or seed coat (Supplementary Fig. S1a, b). The seeds of many *vip4* and *elf7* mutants consistently exhibited abnormal morphology, with a smaller and rounder shape than wild-type seeds at the same stages (Fig. S1), suggesting that the observed ectopic *GUS* expression might be due to pleiotropism in these mutants.

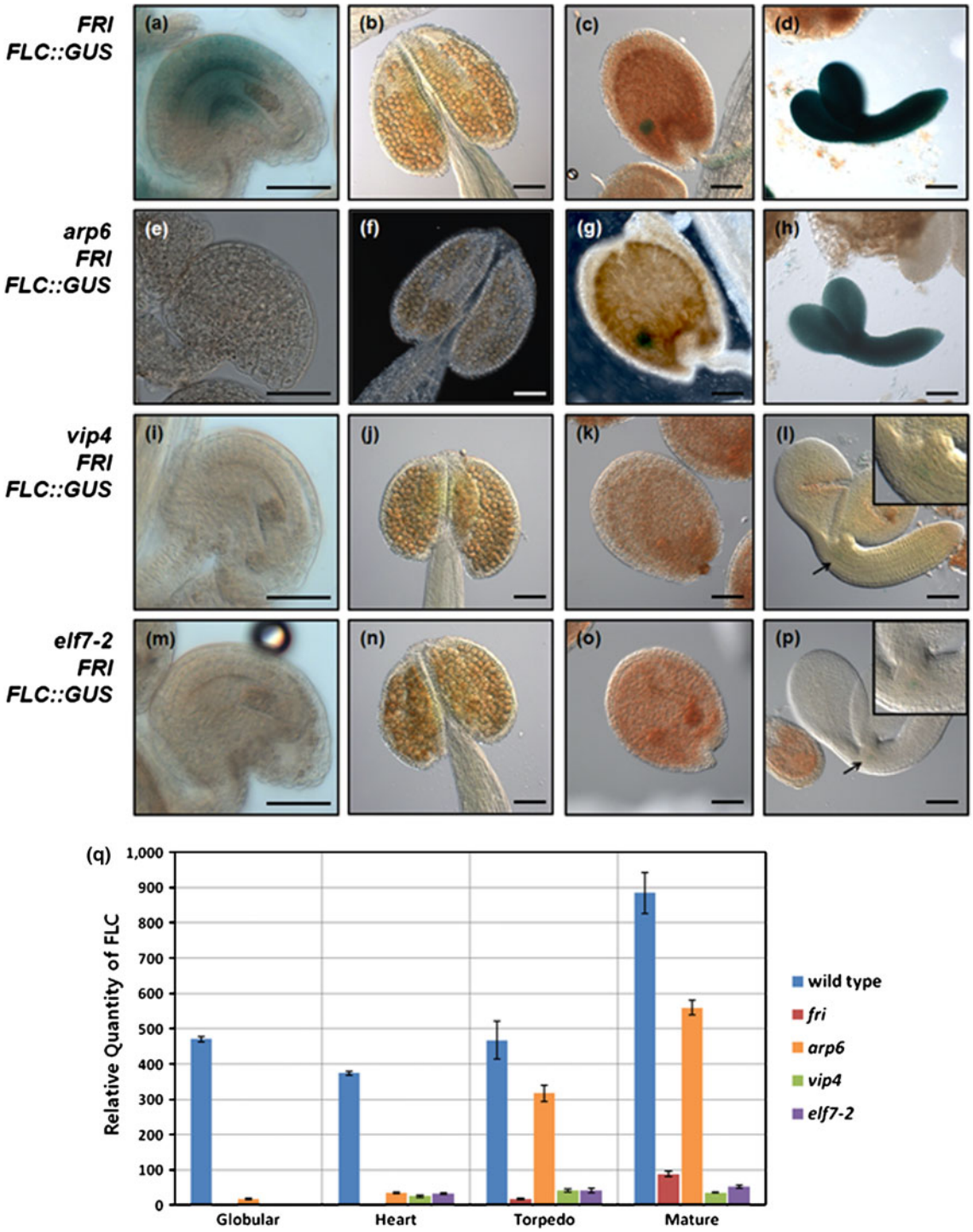

Fig. 3 *FLC::GUS* and *FLC* mRNA expression in *arp6*, *vip4* and *elf7* mutants. *FLC::GUS* expression in the wild-type ovule (**a**), stamen (**b**), developing embryos at the globular stage (**c**), and mature stage (**d**). *FLC::GUS* expression in *arp6* mutant ovule (**e**), stamen (**f**), developing embryos at the globular to heart stage (**g**), and mature stage (**h**). *FLC::GUS* expression in the *vip4* mutant ovule (**i**), stamen (**j**), developing embryos at the globular to heart stage (**k**), and mature stage (**l**). *FLC::GUS* expression in the *elf7* mutant ovule (**m**), stamen (**n**), developing embryos at the globular to heart stage (**o**), and mature stage (**p**). The *arrows* in (**l**) and (**p**) show weak expression in the shoot apical meristem region of *vip4* and *elf7* mutant embryos, respectively, and the *insets* show enlarged images of *FLC::GUS* expression. **q** Real-time qRT-PCR analysis of *FLC* mRNA expression in the developing seeds of wild type and the *fri*, *vip4* and *arp6* mutants. Values are normalized to an internal reference *TUB2* gene and plotted relative to the expression of *FLC* in *fri* globular seeds, which was set to 1.0. Value represents the average of duplicate measurements ± standard error. *Scale bars* ovules, 50 μm; stamens, 100 μm; seeds, 100 μm

We further confirmed the endogenous *FLC* expression by qRT-PCR in *arp6*, *vip4*, and *elf7* mutant seeds. In the *arp6* mutants, *FLC* mRNA was expressed at very low levels in globular and heart seeds (Fig 3q, orange bar). From torpedo to mature stage, *FLC* expression increased but still showed considerably lower than that in the wild type (Fig 3q, orange and blue bar). Therefore, *arp6* mutation causes down-regulation of *FLC* expression throughout embryogenesis as in the *pie1* mutant. Mutations in *vip4* and *elf7* resulted in very low levels of *FLC* mRNA expression throughout embryogenesis (Fig. 3q, green and purple bar). The expression levels in the mature seeds of both mutants were even lower than in the *fri* mutant, demonstrating that a lack of the Paf1-complex causes severe defects and failed to induce *FLC* reactivation in the early stages of embryogenesis, which could not be overcome during the later stages.

Effects of H3K4 methyltransferases in *FLC* reactivation during embryogenesis

SET domain-containing ATX1 and ATXR7 have been characterized as *Arabidopsis* H3K4 methyltransferases that play roles in activating *FLC* (Pien et al. 2008; Tamada et al. 2009) (Fig. 1). To evaluate the potential roles of histone methyltransferases during the reproductive stage, we analyzed *GUS* expression in *atx1* and *atxr7* mutants. Each single mutant showed the same *GUS* expression pattern as *FRI FLC::GUS* plants (Fig. 4a–l). These results indicate that ATX1 and ATXR7 did not affect *FLC::GUS* expression during reproduction, but it is still possible that ATX1 and ATXR7 might have functional redundancy during embryogenesis. Therefore, we generated *atx1 atxr7* double mutants harboring the *FLC::GUS* transgene. However, the *atx1 atxr7* double mutants also did not show the altered *GUS* expression pattern (Fig. 4m–p), indicating that H3K4 methylation by ATX1 and ATXR7 did not have an essential role in *FLC::GUS* transgene reactivation during reproduction. This is in contrast to the previous reports that *atx1* and *atx1 atxr7* mutations resulted in a significant decrease in H3K4 methylation and a concomitant reduction of *FLC* expression, causing an early flowering phenotype similar to the *fri* background (Pien et al. 2008; Tamada et al. 2009). We further investigated *FLC::GUS* expression in F3 seedlings after harvesting seeds. However, the simultaneous absence of *ATX1* and *ATXR7* did not alter the *GUS*-staining pattern (Supplementary Fig. S2).

We also performed real-time qRT-PCR for endogenous *FLC* expression. In *atx1*, *FLC* mRNA was dramatically decreased at the globular stage (Fig. 4q, green bar) but still higher than that in *fri* seeds (Fig. 4q, red bar). From the globular to mature stages, *atx1* plants showed a gradual increase in *FLC* mRNA, and *atx1* mature seeds showed a high *FLC* expression, with a level similar to *FRI*. In contrast, the absence of *ATXR7* had weaker effect than the *atx1* mutation (Fig. 4q, purple bar).

In globular seeds of *atx1 atxr7*mutants, *FLC* expression was highly decreased, similar to that observed in *atx1* mutant seeds (Fig. 4q, orange bar). *FLC* expression in the mature seeds of the double mutant was much lower than that in *FRI* or the single mutants of *atx1* and *atxr7*, but was still higher than that in *fri*. This suggests that ATX1 and ATXR7 H3K4 methyltransferases have functional redundancy on *FLC* chromatin and are required for full reactivation of *FLC* during seed development, although their impacts are weaker than those of the FRI-complex and Paf1-complex.

Effect of DNA methylation on *FLC::GUS* reactivation during reproduction

Regardless of the epigenetic state in adult plants, *FLC* expression is repressed in gametophytes (Choi et al. 2009) (Sheldon et al. 2008). We have previously proposed the possibility that the silencing of *FLC* in gametophytes might be established by the canonical process of epigenetic reprogramming rather than by specific *FLC* repressors (Choi et al. 2009). To examine this possibility, we performed *FLC::GUS* staining in various mutants related to DNA methylation. We chose mutants for the following factors: *DNA METHYLTRANSFERASE 1* (*MET1*), a CG maintenance methyltransferase; *CHROMOMETHYALTRANSFERASE 3* (*CMT3*), a CNG methyltransferase; *DOMAINS REARRANGED METHYLTRANSFERASE 1 and 2* (*DRM1 DRM2*), two *de novo* methyltransferases; and *DECREASED DNA METHYLATION1* (*DDM1*), a SWI1/SNF2 chromatin remodeling ATPase. It has been reported that global DNA hypomethylation occurs in *ddm1* mutant plants (Jeddeloh et al. 1999). We also chose *RPD3-like HISTONE DEACETYLASE 6* (*HDA6*) as a candidate because in the *hda6* mutant, *FLC* expression is up-regulated (Wu et al. 2008), and symmetric DNA methylation is reduced at RNA dependent DNA Methylation (RdDM)-silenced promoters (Aufsatz et al. 2007; Wu et al. 2008). We introduced *FLC::GUS* and *FRI* into *met1 fri*, *cmt3 fri*, *drm1 drm2 fri*, *ddm1 fri*, and *hda6 fri*, and analyzed the *GUS* expression pattern in segregating F2 populations.

Except for the *met1*mutant, all the mutant plants showed *GUS* expression in the sporophytic tissues of the ovules (Fig. 5a, i, m, q, u). The absence of *GUS* expression in *met1* ovules (Fig. 5e) might be a result of the reduction of *FLC* expression in plants with hypomethylation, as previously reported (Finnegan et al. 1998; Genger et al. 2003; Jean Finnegan et al. 2005). However, the down-regulation of *FLC* is not associated with changes in DNA methylation at the *FLC* locus. Instead, antisense *MET1* transgenic

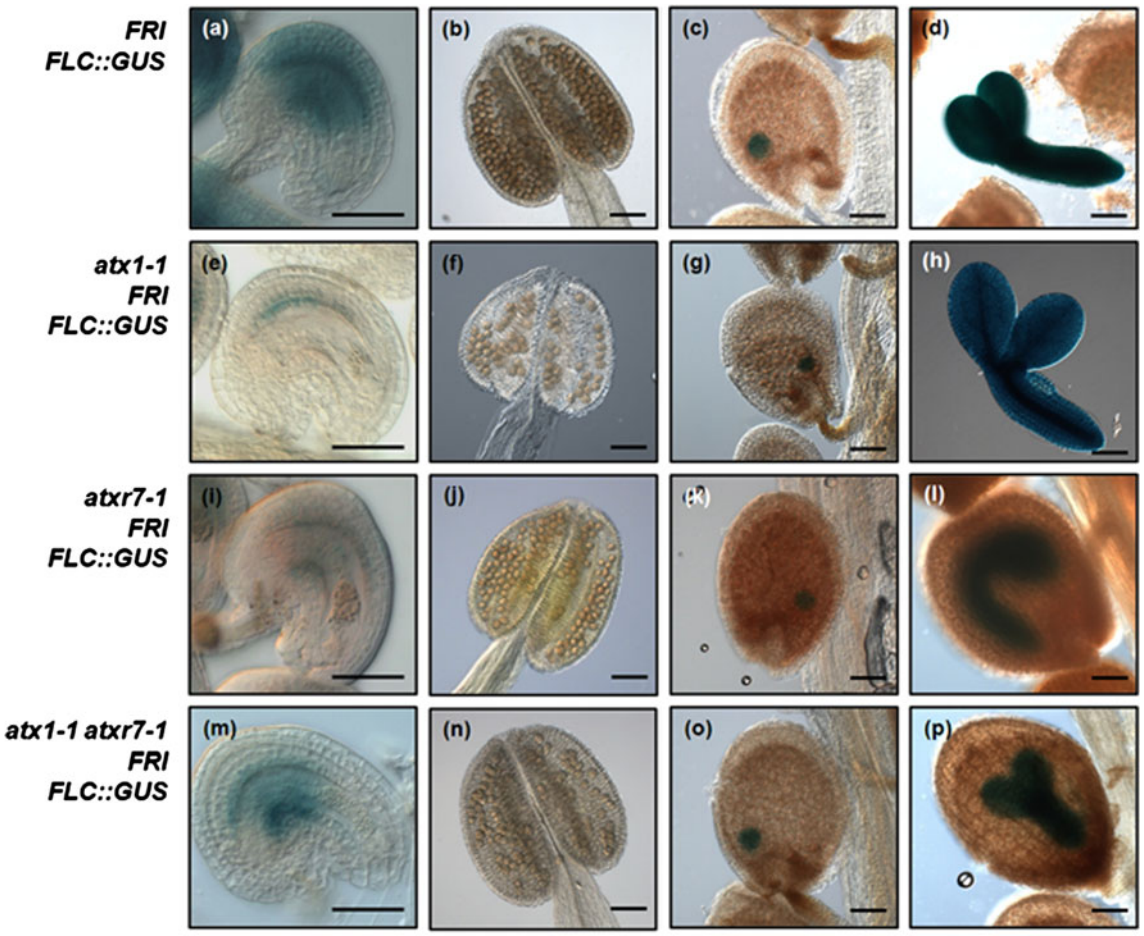

Fig. 4 *FLC::GUS* and *FLC* mRNA expression in H3K4 methyltransferase mutants. *FLC::GUS* expression in the wild-type ovule (**a**), stamen (**b**), developing embryos at the globular stage (**c**), and mature stage (**d**). *FLC::GUS* expression in the *atx1* mutant ovule (**e**), stamen (**f**), developing embryos at the globular stage (**g**), and mature stage (**h**). *FLC::GUS* expression in the *atxr7* mutant ovule (**i**), stamen (**j**), developing embryos at the globular stage (**k**), and mature stage (**l**). *FLC::GUS* expression in the *atx1 atxr7* double mutant ovule (**m**), stamen (**n**), developing embryos at the globular stage (**o**), and mature stage (**p**). **q** Real-time qRT-PCR analysis of *FLC* mRNA expression in the developing seeds of wild type and the *fri*, *atx1, and atxr7* mutants and *the atx1 atxr7* double mutant. Values are normalized to an internal reference *TUB2* gene and plotted relative to the expression of *FLC* in *fri* globular seeds, which was set to 1.0. Value represents the average of duplicate measurements ± standard error. *Scale bars* ovules, 50 μm; stamens, 100 μm; seeds, 100 μm

plants have shown decreases in H3/H4 acetylation and H3K4 methylation at *FLC* the promoter (Jean Finnegan et al. 2005). To compare the DNA methylation states in the *FLC* region in the wild type and the *met1* mutant, we performed PCR amplification after McrBC treatment, which specifically digests methylated DNA (Supplementary Fig. S3). In our results, none of the regulatory sequences in the 9-kb region covering the *FLC* locus were methylated in wild-type rosette leaves. Moreover, the methylation level was unchanged in the *met1-6* null mutant, confirming the indirect role of MET1 on *FLC* expression (Jean Finnegan et al. 2005). None of the mutants showed any *GUS* expression in the embryo sacs or pollen grains that contain the gametophytes (Fig. 5e, f, i, j, m, n, q, r, u, v), suggesting that canonical DNA methylation did not play a role in the gametogenesis-specific *FLC* repression. Rather, low CG DNA methylation can repress *FLC* expression in the sporophyte without alteration in the DNA methylation at the *FLC* locus.

After fertilization, mutations in *met1*, *cmt3*, *ddm1*, and *hda6* showed almost the same *FLC::GUS* pattern as that in *FRI* (Fig. 5c, d, g, h, k, l, o, p, s, t, w, x), indicating that MET1, CMT3, DDM1, and HDA6 are not relevant for *FLC* reactivation or full activation. Interestingly, *FLC::GUS* was not reactivated in the *drm1 drm2* double mutant, and the lack of expression was found throughout embryogenesis (Fig. 5o, p). This raised the possibility that *FLC* cannot be reactivated in *drm1 drm2* mutant. However, endogenous *FLC* level in *drm1 drm2* double mutant by qRT-PCR was slightly decreased but still showed considerable amount compared to other activator mutants during entire embryogenesis (Supplementary Fig. S4a).We reasoned that if the *drm1 drm2* double mutant prevented *FLC* expression, the progenies from these mutants would show decreased expression of endogenous *FLC* and cause early flowering even if they have active *FRI* and *FLC* alleles. Consistent with *GUS* data in seeds, *FLC::GUS* was not expressed in 10 DAG seedlings (Supplementary Fig. S4b). However, the F3 progeny from the *drm1 drm2* mutants did not show an early flowering phenotype (data not shown). We performed semi-quantitative RT-PCR in the seedling of F3 progenies 10 days after germination (DAG) to check the *FLC* mRNA levels. In the *drm1 drm2* mutant, the endogenous mRNA level of *FLC* was not altered, and only *GUS* mRNA expression decreased compared to that in the *FRI* wild type (Supplementary Fig. S4c). Therefore, the lack of *FLC::GUS* reactivation in *drm1 drm2* may be limited to the *FLC::GUS* transgene.

We also performed *GUS* staining in *ddm1 fri* and *hda6 fri* mutants that contains an inactive *fri* allele to check the effect of DNA methylation and histone acetylation on *FLC::GUS* expression in the absence of the FRI-complex. Both of the mutants showed same *GUS* expression patterns during reproductive stage as that in *fri FLC::GUS* plants (Supplementary Fig. S5). In addition, the flowering time of the *ddm1 fri*, *drm1 drm2 fri*, and *cmt3 fri* mutants was not accelerated compared to that of *fri* plants (Supplementary Fig. S6). Taken together, our results suggest that DNA methylation does not play a role in *FLC* reprogramming during the reproductive stage, regardless of the activity of the FRI-complex.

Effect of the siRNA pathway on *FLC* expression during reproduction

DICER-LIKE 3 (DCL3), RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), and NUCLEAR RNA POLYMERASE IVa (NRPD1a) produce 24-nt small RNAs complementary to the 3′ end of *FLC*, and a lack of these small RNAs have caused the up-regulation of *FLC* mRNA levels (Swiezewski et al. 2007). It is, thus, possible that these 24-nt small RNAs might regulate *FLC* reprogramming during the reproductive stage. To examine this possibility, we crossed *rdr1 fri*, *dcl3 fri*, and *ago4* (ARGONAUTE 4) *fri* mutants with *FRI FLC::GUS* plants, and the *FLG::GUS* patterns were analyzed in the F2 population.

None of the siRNA mutants showed gamete-specific *FLC::GUS* expression (Fig. 6e, f, i, j, m, n), indicating that the siRNA pathway was not relevant to the repression of *FLC* during gametogenesis. All of the siRNA mutants showed the same *GUS* expression pattern as the control *FLC::GUS* plants during embryogenesis (Fig. 6g, h, k, l, o, p). siRNA mutants in an inactive *fri* background also showed the same *GUS* expression pattern as the control *fri FLC::GUS* plants (Supplementary Fig. S7). Our data suggest that the siRNA pathway does not participate in the reprogramming of *FLC* during the reproductive stage, regardless of the activity of the FRI-complex.

To examine the effect of miRNA on *FLC* resetting, we generated plants containing the *FLC::GUS* transgene in the *dcl1* mutant with an active *FRI* allele. We observed the same sporophytic *GUS* expression in the ovules of *dcl1* mutants and no expression in gamete cells, suggesting that the miRNA pathway was not involved in *FLC* repression during gametogenesis. Unfortunately, the *dcl1* mutant plant showed severe defects in floral organs (data not shown), and its integument did not develop (Supplementary Fig. S8), therefore, it was impossible for us to analyze the *GUS* expression during embryogenesis due to the lack of viable seeds after fertilization.

Discussion

Here, we present evidence that diverse *FLC* activators play critical roles in the *FLC* reactivation process during

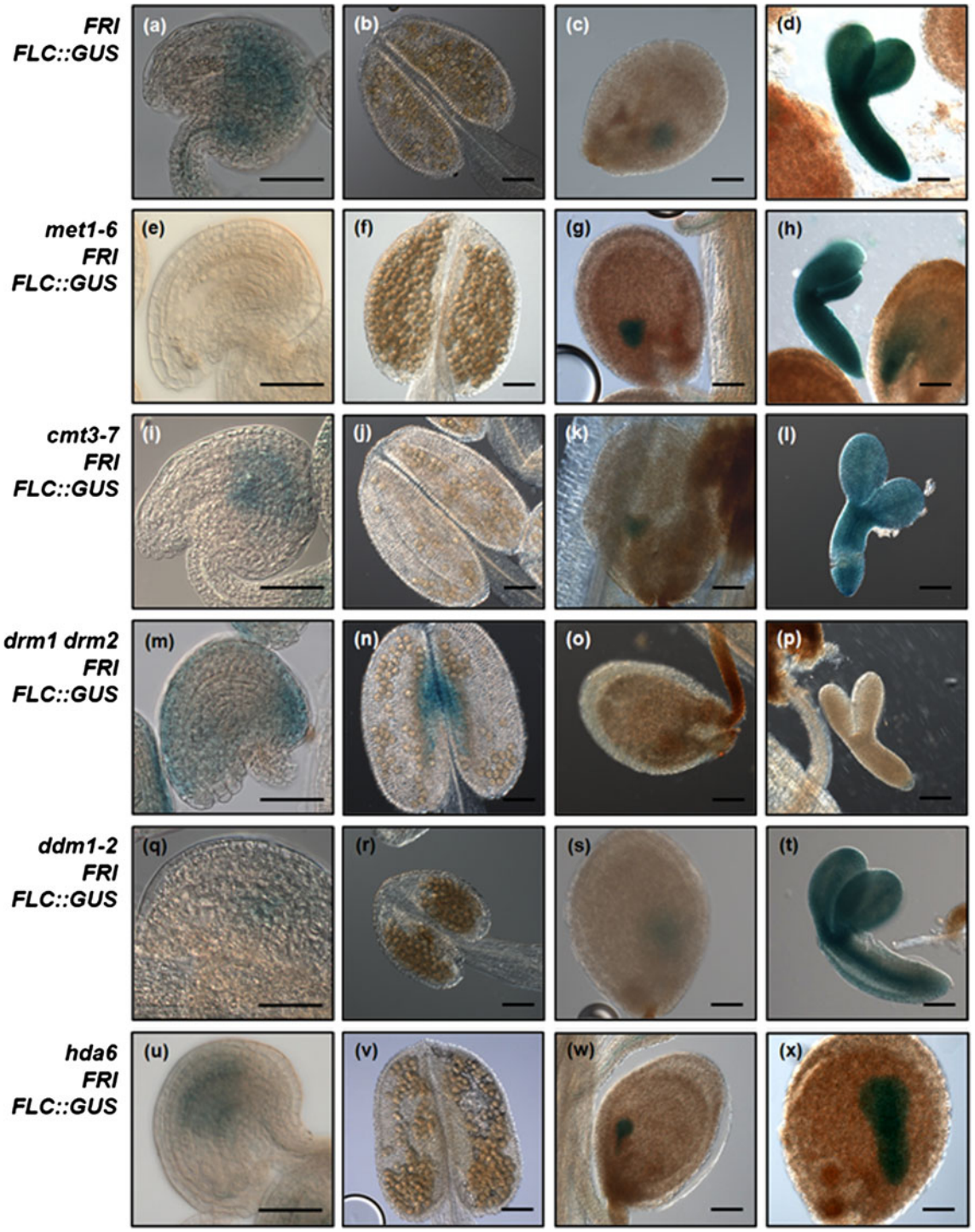

Fig. 5 *FLC::GUS* expression in mutants of the DNA methylation pathway. *FLC::GUS* expression in the wild-type ovule (**a**), stamen (**b**), developing embryos at the globular to heart stage (**c**), and mature stage (**d**). *FLC::GUS* expression in *met1* mutant ovule (**e**), stamen (**f**), developing embryos at the globular to hear stage (**g**), and mature stage (**h**). *FLC::GUS* expression in the *cmt3* mutant ovule (**i**), stamen (**j**), developing embryos at the globular to heart stage (**k**), and mature stage (**l**). *FLC::GUS* expression in the *drm1 drm2* mutant ovule (**m**), stamen (**n**), developing embryos at the globular to heart stage (**o**), and mature stage (**p**). *FLC::GUS* expression in the *ddm1* mutant ovule (**q**), stamen (**r**), developing embryos at the globular to heart stage (**s**), and mature stage (**t**). *FLC::GUS* expression in the *ddm1* mutant ovule (**u**), stamen (**v**), developing embryos at the globular to heart stage (**w**), and mature stage (**x**). *Scale bars* ovules, 50 μm; stamens, 100 μm; seeds, 100 μm

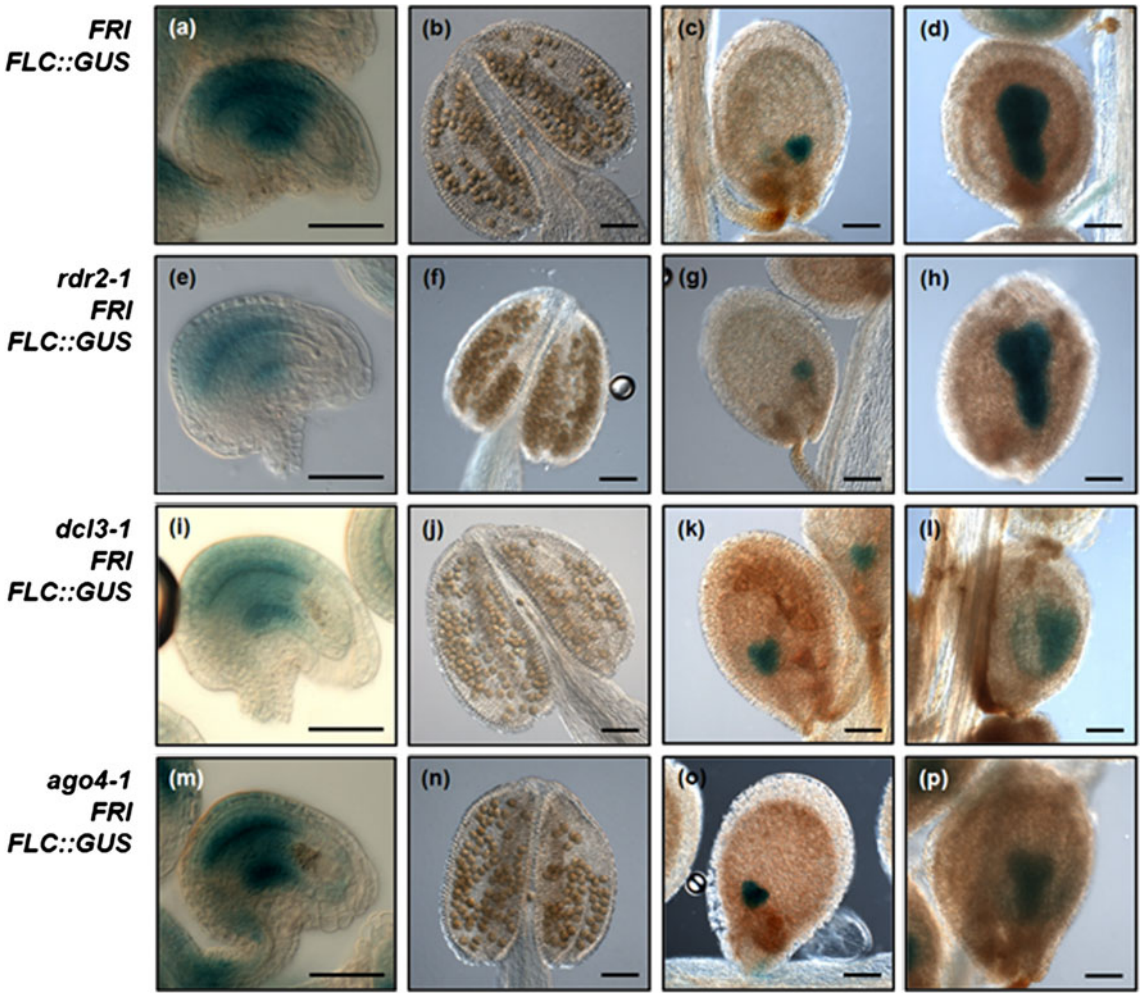

Fig. 6 *FLC::GUS* expression in mutants of the siRNA pathway. *FLC::GUS* expression in the wild-type ovule (**a**), stamen (**b**), developing embryos at the globular to heart stage (**c**), and torpedo stage (**d**). *FLC::GUS* expression in the *rdr2* mutant ovule (**e**), stamen (**f**), developing embryos at the globular to heart stage (**g**), and torpedo stage (**h**). *FLC::GUS* expression in the *dcl3* mutant ovule (**i**), stamen (**j**), developing embryos at the globular to heart stage (**k**), and torpedo stage (**l**). *FLC::GUS* expression in the *ago4* mutant ovule (**m**), stamen (**n**), developing embryos at the globular to heart stage (**o**), and torpedo stage (**p**). *Scale bars* ovules, 50 μm; stamens, 100 μm; seeds, 100 μm

embryogenesis. Mutants of factors in the Paf1-complex and FRI-complex display a significant failure in the full reactivation of *FLC* from young to mature seed development, but *arp6* shows a weaker defect than mutants for the FRI- and Paf1-complexes. *ATX1* and *ATXR7* have redundant roles in *FLC* reactivation, but their effects are weaker than mutants for the FRI- and Paf1-complexes. Our results provide clear evidence that the genetic and epigenetic states for appropriate *FLC* expression in germinated growing plants are already established during embryogenesis by diverse *FLC* regulators.

Function of *FLC* activators in embryogenesis

FLC expression requires various complexes for its activation (Fig 1). The biological function of these complexes has been well studied in the vegetative stage. FRI interacts with FRL1, SUF4, FRIGIDA ESSENTIAL1 (FES1), and FLC EXPRESSOR (FLX), and these proteins comprise the FRI-complex and specifically induce *FLC* expression (Choi et al. 2011). The FRI-complex recruits the chromatin modification factor, the SWR1-complex and a general transcription factor, a TAF14 homolog, for the transcriptional activation of *FLC* (Choi et al. 2011). Similar with the effects in the vegetative tissue, mutations in the FRI-complex components resulted in a significant reduction of the expression level of *FLC* during embryogenesis (Fig. 2s), and the reactivated *FLC* level in *fri* globular seeds was much lower than that in vernalized *FRI* seeds (Fig. 2t). This indicated that the FRI-complex components have critical roles in reactivating *FLC* expression in early embryogenesis, and the defect caused by its deficiency is maintained until seed maturation.

In *Arabidopsis*, it has been reported that mutants of the Paf1-complex show an early flowering phenotype caused by the reduced expression of *FLC* and also show low levels of H3K4 methylation on *FLC* chromatin (He et al. 2004; Oh et al. 2004). This suggests that the *Arabidopsis* Paf1-complex regulates *FLC* by not only transcriptional machinery but also histone modification. The *vip4* and *elf7* mutants almost failed to reactivate *FLC* throughout embryogenesis (Fig. 3q). Because both the FRI-complex and Paf1-complex are bifunctional in the transcriptional process and the histone modification of *FLC* chromatin, it is possible that those complexes play the most critical roles in *FLC* reactivation in our experiments.

In contrast to the FRI- and Paf1-complex, the *arp6* mutant showed a partial decrease of *FLC* expression during seed development (Fig. 3q). ARP6 is a component of the Swr1-complex, which is required for the incorporation of histone variant H2A.Z (Choi et al. 2007; Deal et al. 2007) (Fig. 1). Previously, we have shown that *FLC::GUS* expression was partially decreased in the *pie1* mutant (Choi et al. 2009); thus, it seems that the Swr1-complex has a partial effect in *FLC* reactivation. Given that the Swr1-complex is recruited by the FRI-complex (Choi et al. 2011), it is reasonable to think that the less severe defect by the *arp6* mutation on *FLC* reactivation was due to a hierarchy of complexes. Interestingly, the *atx1 atxr7* double mutant also showed a partial defect in activating *FLC* during embryogenesis (Fig. 4q). It has been reported that FRI recruited WDR5a to the *FLC* locus and that WDR5a interacted with ATX1 in vitro (Jiang et al. 2009). Therefore, ATX1 and ATXR7 might be factors recruited by the FRI-complex, similar to the Swr1-complex.

Conclusively, our results provide evidence that the *FLC* activators that regulate vegetative *FLC* expression already function during embryogenesis. *FLC* reactivation is established by the cooperation of diverse regulators upon fertilization and is maintained during reproduction, which is a prerequisite for the flowering competence of the ensuing generation.

Possible mechanism for *FLC* reprogramming during the reproduction process

In our previous study, *FLC* expression was found to be repressed in gametophytes regardless of the epigenetic state in adult plants (Choi et al. 2009). We also showed that an autonomous-pathway that represses *FLC* expression in the absence of *FRI* is not involved in the repression of *FLC* in gametophyte.

DNA methylation and siRNA are generally associated with gene silencing. CG, CNG and CNN methylation are abundant in the plant genome and function not only in the transcriptional silencing of transposons and repetitive sequences, but also in gene imprinting (Bender 2004; Chan et al. 2005). DNA methylation is maintained by DNA methyltransferase, such as MET1 and CMT3. In addition, DRM2 mediates de novo DNA methylation. The siRNA pathway recruits DRM2 to its target region and causes RdDM (Feng et al. 2010). Because DNA methylation and siRNA, by themselves or working together, participate in gene and transposon silencing during reproduction, we suspected these processes as candidates for the reprogramming of *FLC* expression in the gametophyte. Although key regulators of these mechanisms were used in our experiment, none of them showed a de-regulation of *FLC* both in gametogenesis and in embryogenesis (Figs. 5, 6). Therefore, neither DNA methylation nor siRNA mechanisms function in *FLC* reprogramming during reproductive process, which supports our conclusion that histone-mediated *FLC* regulation might be the basal mechanism of *FLC* reactivation.

In contrast, dynamic chromatin exchange occurs in the primordial germ cells of mice (Hajkova et al. 2008). The erasure of the histone marker and the exchange of histone variants might be involved in this global chromatin dynamic, and this is thought to mediate genomic reprogramming (Hajkova et al. 2008). Similar with this, it has recently been reported that a limited number of H3.3 variants are dominantly present in both female and male germ cells and are actively removed from zygote chromatin in plants (Ingouff et al. 2007, 2010). This raises the possibility of global reprogramming of the epigenetic state by exchange of histone variants during plant reproduction, but this hypothesis requires further studied. Given that *FLC* is not expressed in gametophytes, regardless of the epigenetic state of the adult plants, but is reactivated after fertilization, it is tempting to speculate that the *FLC* epigenetic markers of adult plants are erased and reset sometime during gametogenesis by global histone exchange or by a histone demethylation process. However, the erasure of repressive markers seems insufficient to activate *FLC* without transacting activators. It is intriguing that *FLC* and many activators of *FLC* start to be expressed in the embryos after fertilization during the reproductive phase. It will be interesting to see whether *FLC* can be reactivated during gametogenesis if its activators are expressed in the gamete cells. If so, it will prove the evidence that the epigenetic state of *FLC* in the gametophyte is receptive to activation once activators are induced.

In this study, we addressed the re-activation of *FLC* during embryogenesis in mutants for various epigenetic regulators. Our results contribute to the knowledge of the processes that mediate *FLC* reprogramming for the induction of the appropriate time for flowering.

Acknowledgments We are grateful to S. Michaels for providing the *FLC::GUS* seeds. We thank R. Amasino, R. Fischer, E. Richard, J. Carrington and Arabidopsis Biological Resource Center (ABRC) for providing mutant seeds. This work was supported by grants from Brain Korea 21 program to H. Yun and Y. Hyun, and from the Korea Research Foundation (KRF-2008-314-C00359) to Y. Choi. This work was also supported by National Research Fund Grant 2009-0079227 from the Ministry of Education, Science and Technology Mid-Career Researcher Program Y. Choi.

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