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Resetting and regulation of *FLOWERING LOCUS C* expression during Arabidopsis reproductive development

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Summary

The epigenetic regulation of the floral repressor *FLOWERING LOCUS C* (*FLC*) is one of the critical factors that determine flowering time in *Arabidopsis thaliana*. Although many *FLC* regulators, and their effects on *FLC* chromatin, have been extensively studied, the epigenetic resetting of *FLC* has not yet been thoroughly characterized. Here, we investigate the *FLC* expression during gametogenesis and embryogenesis using *FLC::GUS* transgenic plants and RNA analysis. Regardless of the epigenetic state in adult plants, *FLC* expression disappeared in gametophytes. Subsequently, *FLC* expression was reactivated after fertilization in embryos, but not in the endosperm. Both parental alleles contributed equally to the expression of *FLC* in embryos. Surprisingly, the reactivation of *FLC* in early embryos was independent of FRIGIDA (FRI) and SUPPRESSOR OF FRIGIDA 4 (SUF4) activities. Instead, *FRI*, *SUF4* and autonomous-pathway genes determined the level of *FLC* expression only in late embryogenesis. Many *FLC* regulators exhibited expression patterns similar to that of *FLC*, indicating potential roles in *FLC* reprogramming. An *FVE* mutation caused ectopic expression of *FLC* in the endosperm. A mutation in *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* caused defects in *FLC* reactivation in early embryogenesis, and maintenance of full *FLC* expression in late embryogenesis. We also show that the polycomb group complex components, Fertilization-Independent endosperm and MEDEA, which mediate epigenetic regulation in seeds, are not relevant for *FLC* reprogramming. Based on our results, we propose that *FLC* reprogramming is composed of three phases: (i) repression in gametogenesis, (ii) reactivation in early embryogenesis and (iii) maintenance in late embryogenesis.

Keywords: *FLOWERING LOCUS C*, epigenetic resetting, embryogenesis, gametogenesis, vernalization, *FLC* regulators.

Introduction

The correct timing of flowering is essential for the survival of plant species. Plants have evolved a complex regulatory network that adjusts flowering time in response to various environmental and endogenous signals. *FLOWERING LOCUS C* (*FLC*), a floral repressor, is one of the central regulators of flowering in Arabidopsis (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). *FLC* encodes a MADS domain-containing transcription factor that inhibits the transcription of downstream floral activators. Expression of *FLC* is promoted by FRIGIDA (FRI), and is repressed by sets of genes in the autonomous and vernalization pathways (Sheldon *et al.*, 1999, 2000; Michaels and Amasino, 2001).

The autonomous pathway is composed of a group of genes that repress *FLC* expression in the absence of functional FRI (Sheldon *et al.*, 1999, 2000; Michaels and Amasino, 2001). Among them, *FLOWERING LOCUS D* (*FLD*;

He *et al.*, 2003), *FVE* (Ausin *et al.*, 2004) and *RELATIVE OF EARLY FLOWERING 6* (*REF6*; Noh *et al.*, 2004) encode a lysine-specific demethylase 1 (LSD1) class putative histone demethylase, a homolog of a retinoblastoma-associated protein and a Jumonji domain-containing putative histone demethylase, respectively. These might repress *FLC* transcription via chromatin modification. *FCA* (Macknight *et al.*, 1997), *FPA* (Schomburg *et al.*, 2001), *FY* (Simpson *et al.*, 2003) and *FLOWERING LOCUS K* (*FLK*; Lim *et al.*, 2004) encode RNA-binding or RNA-processing proteins. Although a recent study suggested that FCA and FPA play a role in RNA-dependent chromatin modification (Baurle *et al.*, 2007), the molecular mechanisms by which these proteins repress *FLC* are largely unknown. The putative homeodomain protein LUMINIDEPENDENS (LD; Lee *et al.*, 1994) and the Arabidopsis CREB-binding protein (CBP) homologs HISTONE ACETYLTRANSFERASEs OF THE CBP FAMILY (HACs; Han *et al.*, 2007) are also categorized as autonomous-pathway members. However, the biochemical roles of these proteins in *FLC* repression are not understood.

The late-flowering habit of winter annual Arabidopsis is conferred by dominant alleles of *FRI* and *FLC* (Gazzani *et al.*, 2003; Michaels *et al.*, 2003). FRI, a protein with two coiled-coil domains, elevates *FLC* expression, even in the presence of the autonomous-pathway repressors (Johanson *et al.*, 2000). SUPPRESSOR OF FRIGIDA 4 (SUF4), a C2H2-type zinc-finger protein, was recently characterized as an interacting partner of FRI (Kim *et al.*, 2006). SUF4 binds to the *FLC* promoter, and might recruit a protein complex containing FRI to activate *FLC*. Arabidopsis homologs of the members of yeast RNA polymerase II-associated factor 1 (PAF1) complex, EARLY FLOWERING 7 (ELF7), also known as VERNALIZATION INDEPENDENCE 2 (VIP2), ELF8 (VIP6), VIP4 and VIP5, are required for elevated *FLC* expression (He *et al.*, 2004; Oh *et al.*, 2004; Kim *et al.*, 2005). EARLY FLOWERING IN SHORT DAYS (EFS), also known as SET DOMAIN GROUP 8 (SDG8), a SET-domain containing putative histone methyltransferase, is also required for *FLC* activation, and either trimethylation at histone H3 lysine 4 (H3K4; Kim *et al.*, 2005) or dimethylation at histone H3 lysine 36 (H3K36; Zhao *et al.*, 2005) in *FLC* chromatin. Thus, mutations in these genes prevent the expression of *FLC* in both *FRI*-containing winter annuals and in autonomous-pathway mutants. In addition, PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1; Noh and Amasino, 2003), SUPPRESSOR OF FRIGIDA 3/ACTIN-RELATED PROTEIN 6/EARLY IN SHORT DAYS 1 (SUF3/ARP6/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 Arabidopsis homologs of members of the yeast SWR1 complex. This complex mediates the exchange of histone protein H2A with its variant H2A.Z, and this process is required for the full activation of *FLC* (Choi *et al.*, 2007).

Vernalization establishes competence for flowering in winter annuals after the prolonged cold of winter (Sung and Amasino, 2005). Vernalization leads to a series of repressive modifications in *FLC* chromatin, and this repression of *FLC* permits the photoperiod pathway to accelerate flowering. The transcriptional induction of *VERNALIZATION INSENSITIVE 3* (*VIN3*) is an initial step in vernalization-induced *FLC* repression (Sung and Amasino, 2004). Expression of the PHD domain protein VIN3 is necessary for the deacetylation of histone H3, and the methylation at histone H3 lysine 9 (H3K9) and histone H3 lysine 27 (H3K27) within *FLC* chromatin during cold treatment. After this, the B3-domain protein VERNALIZATION 1 (VRN1) and the polycomb group protein VERNALIZATION 2 (VRN2) maintain the repressed state of *FLC* chromatin (Gendall *et al.*, 2001; Levy *et al.*, 2002; Bastow *et al.*, 2004; Sung and Amasino, 2004). Subsequently, vernalization-mediated *FLC* repression is stably maintained under warm conditions. However, this 'memory of winter' is reset in the next generation, and this reprogramming is critical to reestablish the vernalization requirement each generation.

Various studies have focused on isolating *FLC* regulators and understanding how they regulate *FLC* transcription in the post-embryonic vegetative developmental stages. In contrast, less is known about the resetting of *FLC* during reproductive development. In this work, we have studied the expression patterns of *FLC* and a variety of *FLC* regulators, and have determined the effects of *FLC* regulators on *FLC* expression during reproductive development. Our results indicate the existence of an epigenetic reprogramming of gene expression, which takes place during gametogenesis and embryogenesis, in flowering plants that is analogous to that in mammals.

Results

Reprogramming of FLC *expression during gametogenesis and embryogenesis*

To explore the resetting of *FLC*, the spatiotemporal expression pattern of *FLC::GUS* was analyzed in gametophytes and developing embryos of *FLC::GUS FRI flc-3* plants in the Columbia-0 (Col-0) ecotype background. In the *FLC::GUS* construct, the *GUS* gene was inserted in frame into an *Nhe*I site located in the sixth exon of a 16-kb genomic clone, spanning 5.4-kb upstream of the *FLC* start site and 5-kb downstream of the stop codon (Michaels *et al.*, 2005). Before fertilization, *FLC::GUS* expression was detected in ovules, but not in stamens, of non-vernalized plants (Figure 1a,b). The region showing GUS staining in the ovule was restricted to the central cell of the embryo sac, and the part of the integument that originates from sporophytic maternal tissue. To examine whether the GUS signal in the ovule was a result of gametophytic expression *per se*, or whether it was

Figure 1. Reprogramming of *FLC* during gametogenesis and embryogenesis.
(a and b) *FLC::GUS* expression in female and male gametophytes of non-vernalized (NV) plants prior to fertilization. (c and d) *FLC::GUS* expression in female and male gametophytes of vernalized (V) plants prior to fertilization. (e–i) *FLC::GUS* expression in developing seeds of non-vernalized plants. (j–n) *FLC::GUS* expression in developing seeds of vernalized plants. (a and c) Ovules; (b and d) stamens; (e and j) early globular stage; (f and k) globular stage; (g and l) heart stage; (h and m) torpedo stage; (i and n) walking-stick stage. Plants used for GUS analysis are of the *FLC::GUS FRI flc-3* genotype in the Col-0 background. (o) Endogenous expression of *FLC* mRNA in various tissues, including gametophytes and developing seeds, from non-vernalized and vernalized plants. RT-PCR analysis was performed with total RNA extracted from different stages and tissues of *FRI FLC* plants in the Col-0 background. To analyze *FLC* expression in developing seeds, seeds containing embryos at each stage were independently harvested for total RNA extraction. *Actin2* (*ACT2*) was used as a quantitative expression control. Ovules include integuments and placentas. (p) Quantitative comparison of *FLC* expression between seeds from plants with or without vernalization treatment. Real-time qPCR was performed using the same cDNAs as in (o). The vertical axis represents the normalized relative expression of *FLC* in comparison with that of *Tubulin2* (*TUB2*). See Experimental procedures for the details. Scale bars: ovules, 20 μm; stamens, 100 μm; seeds, 100 μm.

of sporophytic origin, we generated *FLC::GUS* hemizygous plants by reciprocal crosses between *FLC::GUS* homozygotes and wild-type (WT) plants. Given that only half of the female gametophytes of the *FLC::GUS* hemizygous plants contained the transgene, if the GUS signal originated from the female gametophytes *per se*, it would be detected in half of the ovules. However, all of the ovules in the hemizygous plants exhibited a GUS signal (Figure S1), indicating that *FLC::GUS* expression in the ovules was derived from diploid maternal tissues, and not from gametophytic embryo sacs.

After fertilization, the GUS signal began to appear in embryos from the early globular stage, and was sustained throughout the rest of embryonic development (Figure 1e–i). A weak GUS signal in the endosperm was also detected immediately after fertilization (Figure 1e), but this is likely to have resulted from the residual expression of *FLC::GUS* in the maternal tissues of the ovule, because all of the seeds from *FLC::GUS* hemizygous plants displayed this expression pattern in the endosperm immediately after fertilization (data not shown).

To determine which parental allele of *FLC* contributes to expression in embryos, and to test for the possibility of imprinting, we introduced the *FLC::GUS* transgene uniparentally by reciprocal crosses between *FLC::GUS* and WT plants, and then compared the resulting GUS expression patterns (Figure 2). Paternally and maternally inherited *FLC::GUS* transgenes showed the same expression patterns in developing embryos. Thus, *FLC* is not imprinted, and both parental alleles contribute equally to expression in embryos.

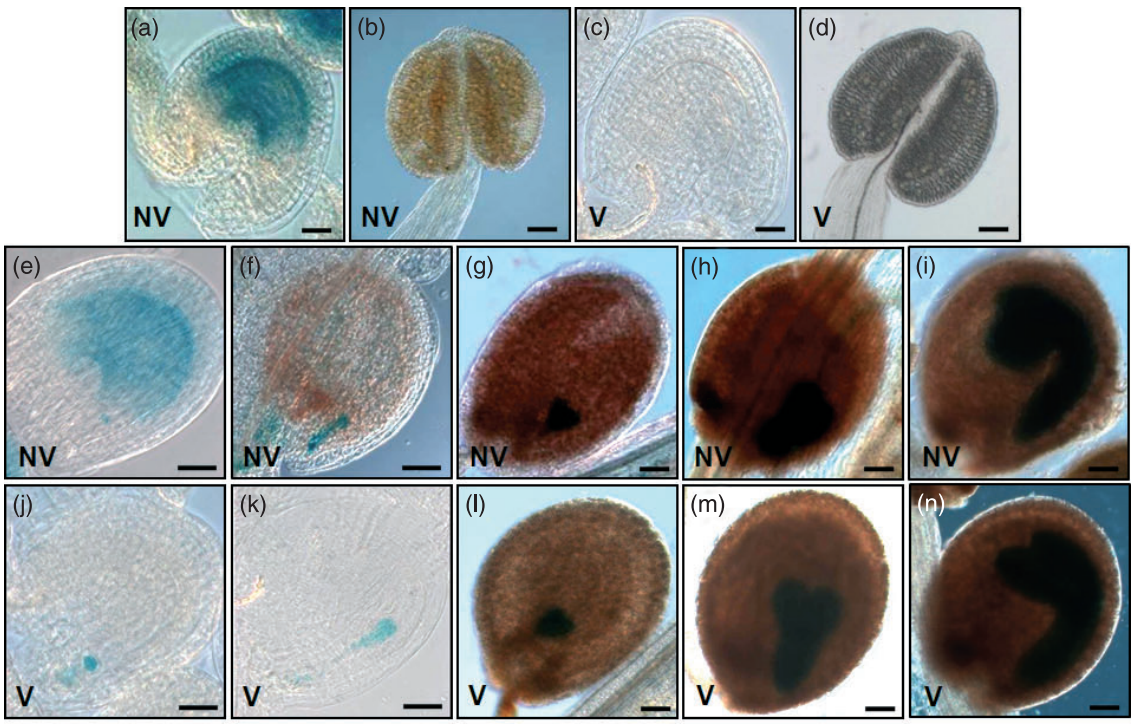
We also performed semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis to examine whether the expression of endogenous *FLC* has the same pattern as that of the *FLC::GUS* transgene (Figure 1o). We observed ubiquitous expression of *FLC* in vegetative tissues, and its expression reached the highest levels in inflorescence meristem, including floral buds. Consistent with the GUS staining results, endogenous *FLC* mRNA was detected in ovules prior to fertilization, and was expressed throughout embryogenesis. However, the expression of *FLC* mRNA in unfertilized ovules should originate from maternal diploid cells, as described above. Taken together, these results indicate that *FLC* expression is repressed during gametogenesis, and is then reactivated after fertilization in embryos.

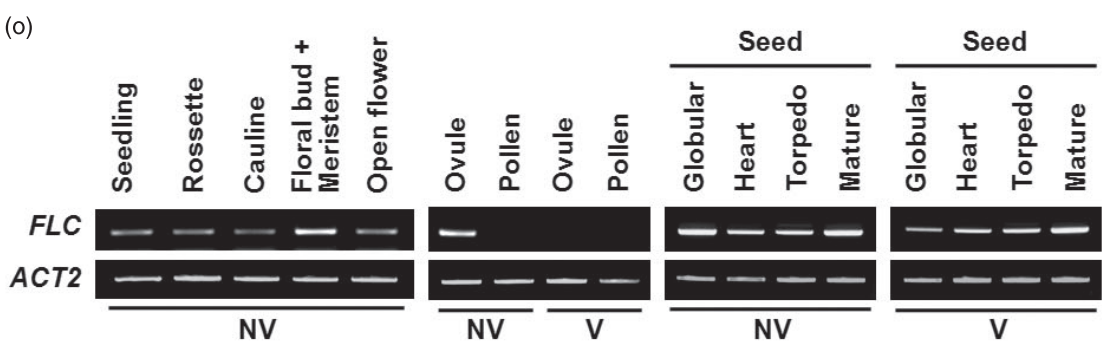
Reprogramming of the vernalization-induced silencing of FLC *during reproductive development*

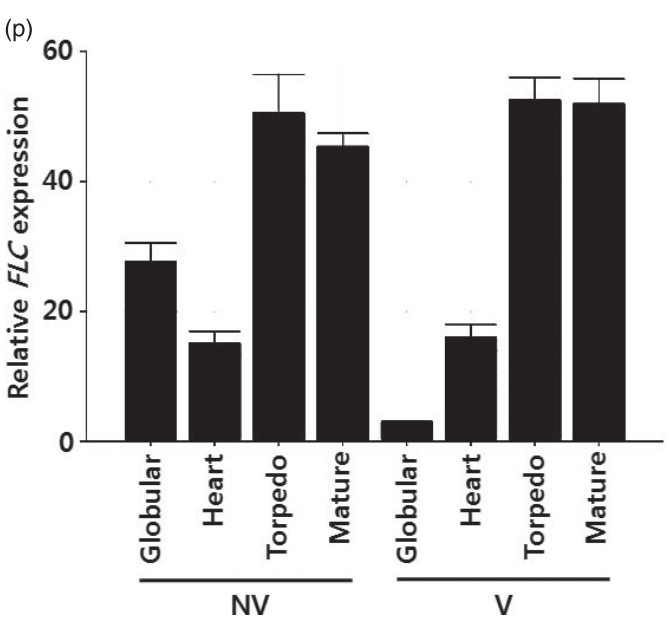
To characterize the resetting of *FLC* after vernalization, we studied the expression pattern of *FLC* by RT-PCR, as well as by using the *FLC::GUS* transgene. *FLC* mRNA was not detected by RT-PCR in ovules or pollens of vernalized plants (Figure 1o), which is consistent with *FLC::GUS* expression (Figure 1c,d). Seeds from vernalized plants exhibited similar *FLC* expression pattern as those from non-vernalized plants throughout the embryonic stages, as analyzed by RT-PCR (Figure 1o) or by using the *FLC::GUS* transgene (Figure 1j–n). We observed a lower *FLC* mRNA level in seeds with globular-stage embryos after vernalization, which is presumably to the result of vernalization-induced repression of *FLC* in maternal tissues within the ovules. Consistent with this hypothesis, seeds from vernalized *FLC::GUS* plants did not show residual GUS expression at early embryonic stages (compare Figure 1j with 1e). When we used random decamer primers instead of an oligo-dT primer for RT, we observed a similar expression pattern of *FLC* mRNA throughout the embryonic stages in vernalized seeds (data not shown), indicating that *FLC* mRNA is not subject to poly(A)-tail-mediated stability control during embryogenesis. Real-time quantitative RT-PCR (real-time qPCR) was also employed to compare *FLC* expression between seeds from plants with or without vernalization (Figure 1p). Similar to the above results, globular stage seeds from vernalized plants exhibited a lower *FLC* expression level than those from non-vernalized plants. *FLC* expression in seeds was similarly increased in both samples after the globular stage. In summary, our results demonstrate that, regardless of the epigenetic state of maternal tissues, *FLC* expression is repressed in gametophytes and is then reactivated in embryos, but not in endosperms, after fertilization. This epigenetic reprogramming of *FLC* expression is similar to that of mammalian systems, in which epigenetic markers are erased and reset during reproductive development (Reik *et al.*, 2001).

Expression of FLC *activators during reproductive development*

FLC is regulated by a number of factors in various floral regulatory pathways, such as *FRI*, vernalization and the







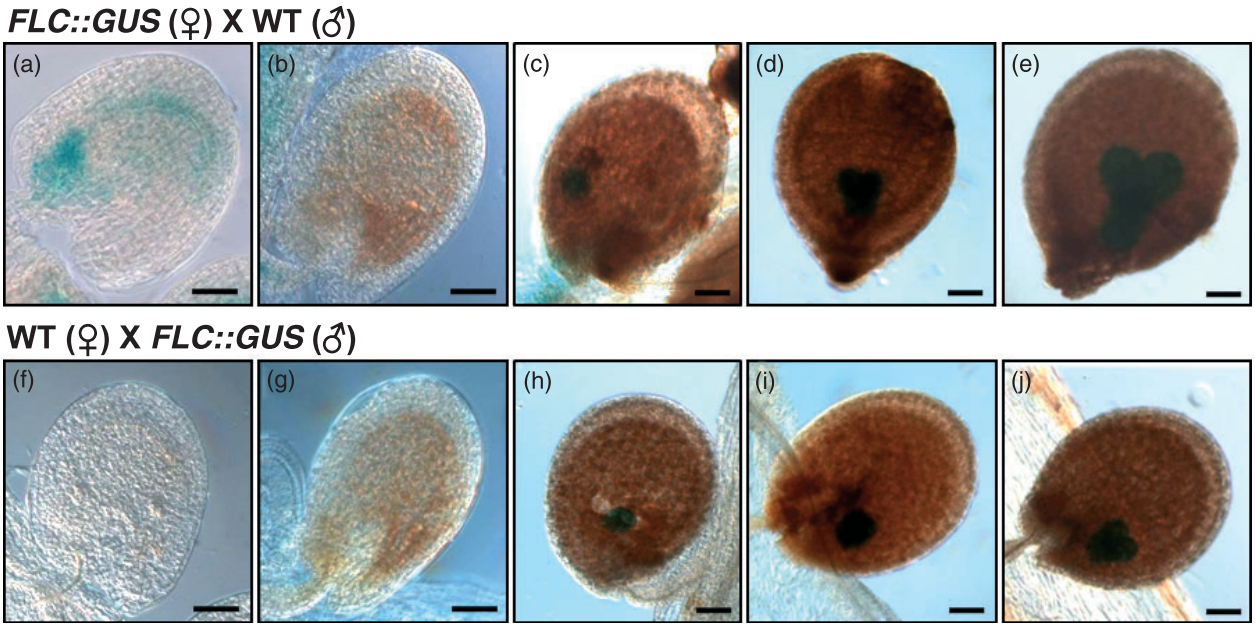


Figure 2. *FLC::GUS* expression in embryos produced by reciprocal crosses between wild-type (WT) and *FLC::GUS* plants.
(a–e) *FLC::GUS* expression in F_1 seeds of *FLC::GUS* (♀) × WT (♂). (f–j) *FLC::GUS* expression in F_1 seeds of WT (♀) × *FLC::GUS* (♂). (a and f) One day after pollination (DAP); (b and g) 3 DAP; (c and h) 5 DAP; (d and i) 7 DAP; (e and j) 9 DAP. Scale bars: 100 μm.

autonomous pathway (Sheldon *et al.*, 1999, 2000; Michaels and Amasino, 2001; Baurle and Dean, 2006). To evaluate whether these factors might also play roles in *FLC* reprogramming during reproductive development, we examined the mRNA expression patterns of several *FLC* activators, including *FRI*, *EFS*, *PIE1* and *ELF7*, by RT-PCR. As shown in Figure 3a, these genes displayed ovule-specific expression prior to fertilization. After fertilization, these *FLC* activators were constitutively expressed from the globular to the mature embryonic stages. Vernalization had little effect on the expression of these genes during gametogenesis and embryogenesis (Figure 3a).

The expression patterns of *FRI*, *PIE1* and *EFS* were further analyzed using transgenic plants with *GUS* fusion constructs (Figure 3b). Like the *FLC* promoter, the *FRI*, *PIE1* and *EFS* promoters also drove GUS expression in ovules, but not in stamens. The ovule-specific expression of these genes arose from maternal diploid cells, and not from gametophytic cells, because all of the ovules of hemizygous transgenic plants containing the *GUS* fusion constructs were stained, as they were for *FLC::GUS* hemizygous plants (data not shown). There was minor variation in the ovule-specific expression of these genes: FRI_{pro}*::FRI:GUS* and *EFS::GUS* were expressed preferentially in the central region of ovule, whereas *PIE1::GUS* was expressed in the chalazal end of ovule, which is connected to the funiculus (Figure 3b). Despite these differences during gametogenesis, all of these genes exhibited embryo-specific promoter activity during embryogenesis. Taken together, these results suggest that genes acting as *FLC* activators in vegetative development are expressed similarly to *FLC* during embryogenesis.

Expression of FLC *repressors during reproductive development*

To gain insight into the potential roles of *FLC* repressors in the reprogramming of *FLC*, their expression patterns during gametogenesis and embryogenesis were analyzed. We first performed RT-PCR analysis of the expression of the autonomous-pathway genes *FVE*, *FLD*, *LD*, *FCA*, *FY*, *FPA*, *HAC1*, *HAC12*, *FLK* and *REF6*, either with or without vernalization treatment (Figure 4a). Expression of *FVE* and *FLD* mRNA was detected neither in ovules nor in pollen. After fertilization, *FVE* was strongly reactivated during embryogenesis, whereas *FLD* was expressed at very low levels in developing seeds. Expression of *LD* mRNA was low and ovule-specific prior to fertilization, but increased strongly in developing seeds. In addition to expression in ovules and seeds, *FCA*, *FY*, *FPA*, *HAC1* and *HAC12* showed weak but significant expressions in pollen. In contrast, *FLK* and *REF6* did not show expression patterns that were different from *FLC*. We also analyzed the expression of the vernalization pathway component *VIN3* (Figure 4a). Interestingly, *VIN3* expression was weak in ovules and seeds, but strong in pollen grains. *VIN3* mRNA was also present in the non-vernalized seeds. Because *VIN3* expression is barely detectable in vegetative tissues without vernalization (Sung and Amasino, 2004), this result indicates the existence of a mechanism for *VIN3*

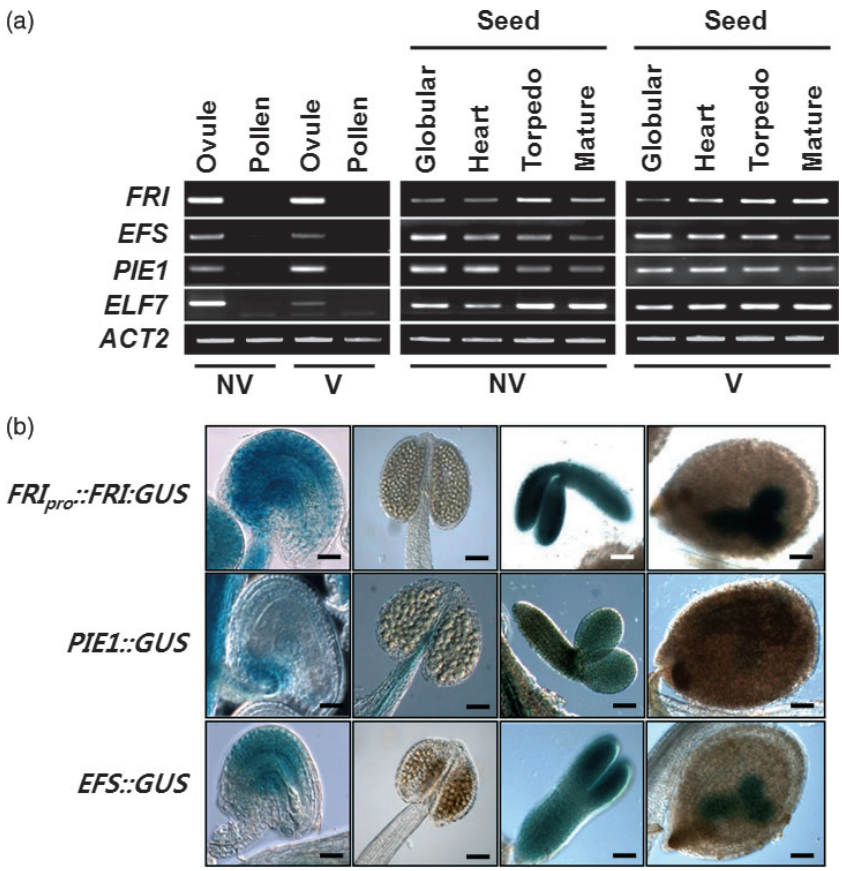


Figure 3. Expression of *FLC* activators during gametogenesis and embryogenesis.
(a) Endogenous mRNA expression of *FLC* activators. Total RNA was isolated as described in Figure 1o. *ACT2* was used as a quantitative expression control.
(b) GUS expression in gametophytes and developing seeds of transgenic plants containing *GUS* fusions for each *FLC* activator. Either the promoters (*PIE1* and *EFS*) or the coding region along with the promoter (*FRI*) were used to drive GUS expression. All transgenic plants except for *PIE1::GUS* (Ws) were in the Col-0 (*fri FLC*) genetic background. Left to right: ovule, stamen, walking-stick to mature embryo, and whole seed with endosperm and embryo. Scale bars: ovules, 20 μm; stamens, 100 μm; embryos, 100 μm; seeds, 100 μm.

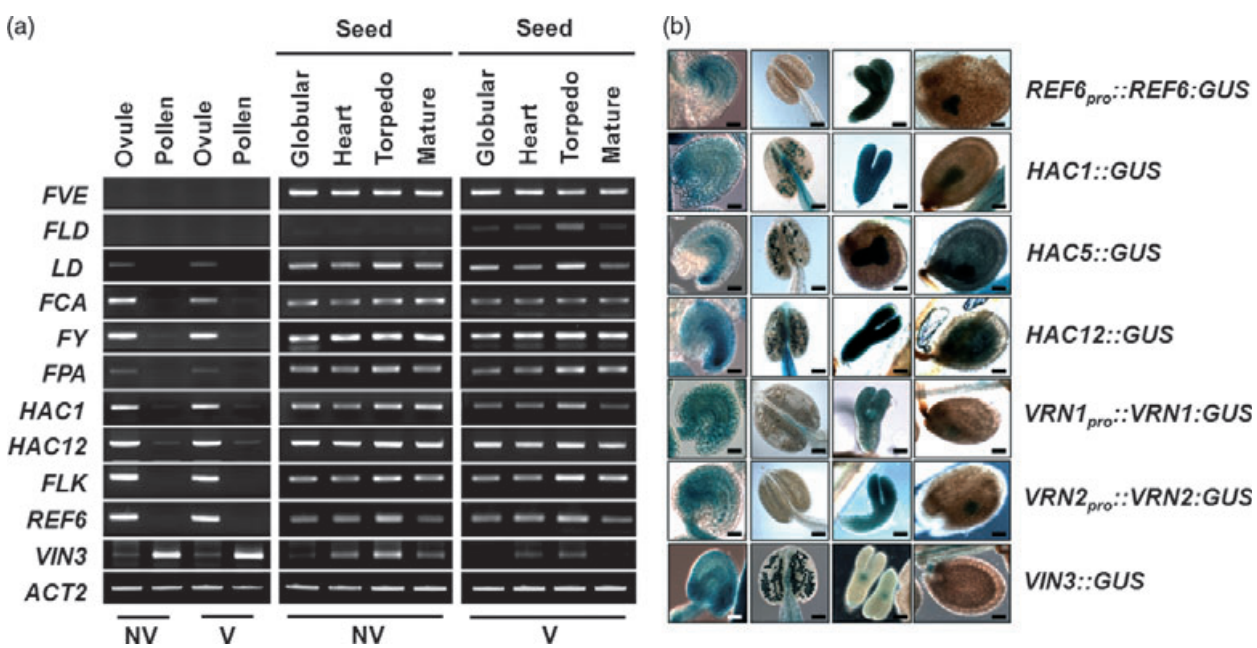


Figure 4. Expression of *FLC* repressors during gametogenesis and embryogenesis.
(a) Endogenous mRNA expression of *FLC* repressors. Total RNA was isolated as described in Figure 1o. *ACT2* was used as a quantitative expression control.
(b) GUS expression in gametophytes and developing seeds of transgenic plants containing *GUS* fusions of each *FLC* repressor. Either the promoters (*HAC1*, *HAC5*, *HAC12* and *VIN3*) or the coding regions along with the promoters (*REF6*, *VRN1* and *VRN2*) were used to drive GUS expression. All transgenic plants except for $VRN1_{pro}$::*VRN1:GUS* and $VRN2_{pro}$::*VRN2:GUS* (*Ler*) were in the Col-0 (*fri FLC*) genetic background. Left to right: ovule, stamen, walking-stick to mature embryo, whole seed with endosperm and embryo. Scale bars: ovules, 20 μm; stamens, 100 μm; embryos, 100 μm; seeds, 100 μm.

regulation in reproductive tissues, that differs from that in vegetative tissues.

The expression levels and patterns of most of the autonomous-pathway genes examined above, during reproductive and embryonic development, were not affected by vernalization treatment during vegetative growth (Figure 4a). Interestingly, unlike other *FLC* repressors in the autonomous pathway, *FLD* and *VIN3* expression levels in the seeds of vernalized plants were slightly higher and lower than those in the seeds of non-vernalized plants, respectively.

The expression patterns of *REF6*, *HAC1*, *HAC5*, *HAC12*, *VRN1*, *VRN2* and *VIN3* were further studied by histochemical GUS assays with transgenic plants harboring transcriptional or translational *GUS* fusion constructs (Figure 4b). Like *FLC::GUS*, $REF6_{pro}$*::REF6:GUS* was specifically expressed in ovules and embryos. *HAC1::GUS*, *HAC5::GUS* and *HAC12::GUS* were expressed in pollen grains. These genes were also expressed in ovules, but the regions expressing GUS were somewhat different from each other. Whereas *HAC1::GUS* was expressed in the entire ovule, expression of *HAC5::GUS* or *HAC12::GUS* was more concentrated in regions containing the egg cell and the central cell. Interestingly, *HAC5::GUS* and *HAC12::GUS*, but not *HAC1::GUS*, were expressed not only in embryos but also in endosperms.

We also studied the expression pattern of some key *FLC* repressors acting in the vernalization pathway. The $VRN1_{pro}$*::VRN1:GUS* and $VRN2_{pro}$*::VRN2:GUS* constructs, which nearly completely rescue the *vrn1* and *vrn2* mutant phenotypes, respectively (CL and CD, unpublished data), showed expression patterns similar to *FLC::GUS* during gametogenesis and embryogenesis (Figure 4b). However, the pattern of *VIN3* expression was different from those of *FLC*, *VRN1* and *VRN2*. *VIN3::GUS* was expressed both in ovules and pollen prior to fertilization. In addition, unlike most of the *FLC* regulators tested in this study, which are expressed in the entire embryo, *VIN3::GUS* expression was restricted to the shoot apical meristem region of the embryo (Figure 4b). Taken together, our results suggest that the majority of *FLC* repressors have expression patterns similar to *FLC* with a few exceptions, namely, *HAC1*, *HAC5*, *HAC12* and *VIN3*, which are also expressed in pollen and/or in the endosperm, where *FLC* is not expressed. These results suggest the possibility that repression of *FLC* in gametophytes and the endosperm might be mediated by the *FLC* repressors that are expressed in those tissues.

Reprogramming of FLC *in the mutant backgrounds of* FLC *regulators*

The expression analysis of *FLC* regulators suggested their potential roles in the reprogramming of *FLC* during gametogenesis and embryogenesis. To identify factors mediating *FLC* reprogramming, we studied the expression of *FLC* in mutants of various *FLC* regulators. First, we introduced the *FLC::GUS* transgene into *fld*, *ld* and *fve* mutants by genetic crosses, and analyzed the resulting GUS expression patterns (Figure 5a). The *fld* and *ld* mutations did not alter the expression pattern of *FLC::GUS* during gametogenesis and embryogenesis, indicating that the reprogramming of *FLC* is independent of the functions of FLD and LD. In contrast, *FLC::GUS* was expressed in the endosperm as well as in the embryo in *fve* mutants (Figure 5a). The ectopic expression of *FLC* in the endosperm was not apparent immediately after fertilization, but began to be detectable in seeds containing torpedo-stage embryos (Figure S2).

HAC1, *HAC5* and *HAC12* are expressed in pollen, and *HAC5* and *HAC12* are also expressed in the endosperm (Figure 4). Therefore, we tested whether *FLC* is ectopically expressed in pollen or the endosperm in single or double *hac* mutant backgrounds by RT-PCR analysis. *FLC* expression was higher in *hac1* single and *hac1 hac12* double mutants in open flowers than in controls (Figure S3a). However, there was no ectopic expression of *FLC* in these *hac* mutants in pollen. *FLC* expression was not increased in the pollen, endosperm or embryos of *hac5 hac12* double mutants (Figure S3b).

Vernalization induces the transcriptional activation of *VIN3* (Sung and Amasino, 2004), and increased *VIN3* expression results in the epigenetic repression of *FLC* in vegetative tissues. Our results showing the strong expression of *VIN3* in pollen grains (Figure 4a,b) also suggest a possible role for VIN3 in the repression of *FLC* in male gametophytes. However, in *vin3* mutants, *FLC::GUS* expression was not detected in pollen, and the expression pattern of *FLC* during gametogenesis and embryogenesis was unchanged (Figure 5a). The results of *FLC::GUS* expression analysis in the *hac* and *vin3* mutants indicate that these genes are not involved in *FLC* repression in pollen and the endosperm, nor are they involved in the reprogramming of *FLC* during gametogenesis and embryogenesis.

Because *FRI*, a transcriptional activator of *FLC*, is expressed in a similar pattern as *FLC* during gametogenesis and embryogenesis (Figure 3), we tested whether FRI functions in *FLC* reactivation upon fertilization. To achieve this, we generated *FLC::GUS fri flc* plants, and compared their GUS expression patterns with those of *FLC::GUS FRI flc* plants during embryogenesis (Figure 5b). Interestingly, the early stage embryos of the *fri* plants showed strong GUS expression, similar to GUS expression in the same stage embryos of *FRI* plants. However, after the late-torpedo embryonic stages, GUS expression in the *fri* plants decreased gradually, and eventually was almost fully repressed in mature embryos, with minor expression in the vasculature. In contrast, GUS was strongly expressed in the *FRI* plants until embryonic maturation, and was then

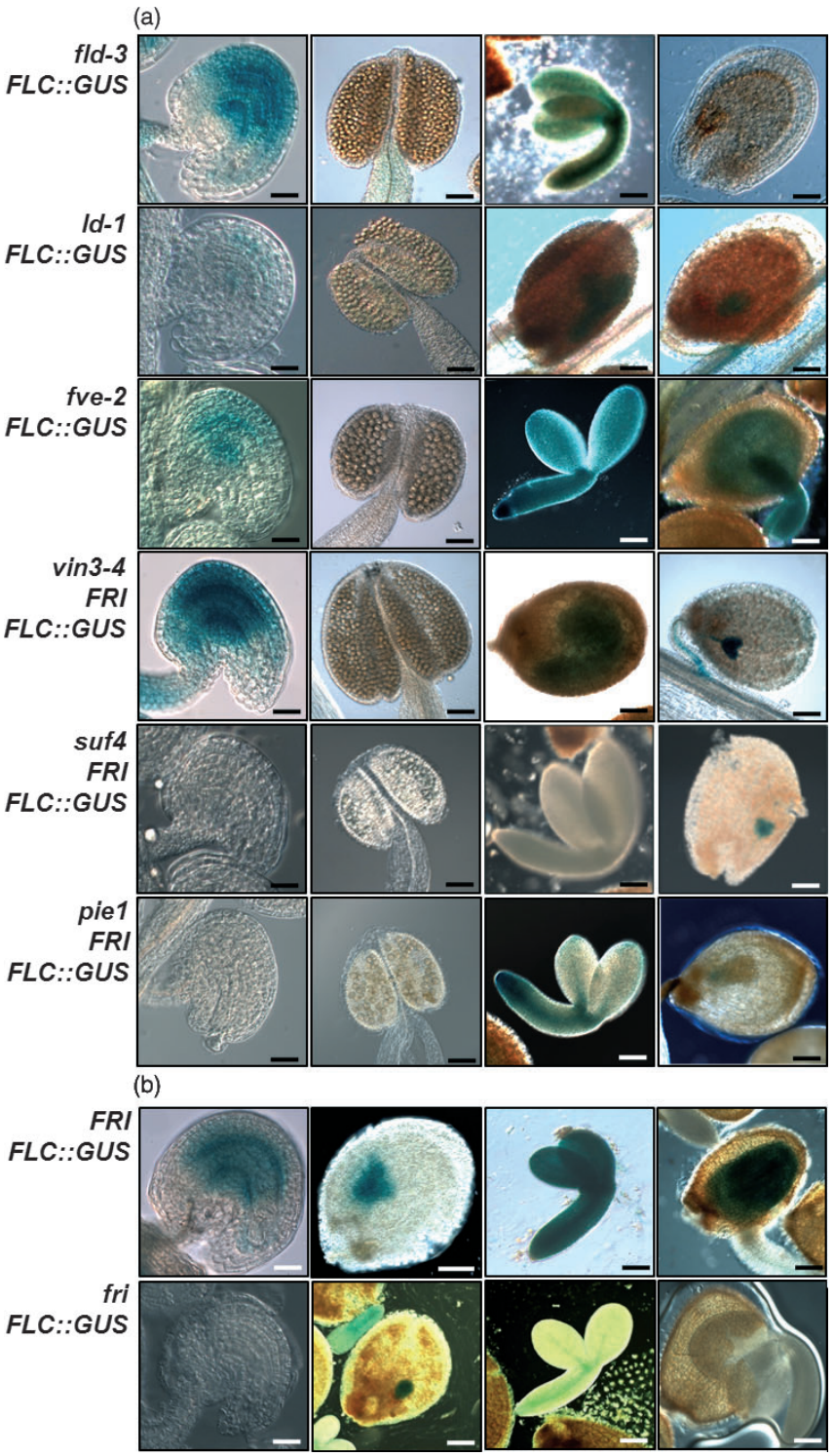


Figure 5. *FLC::GUS* expression in mutants of *FLC* regulators.
(a) *FLC::GUS* expression in mutants of *FLC* repressors and *FLC* activators. Left to right: ovule, stamen, walking-stick to mature embryo, and whole seed with endosperm and embryo.
(b) *FLC::GUS* expression in developing embryos in the *FRI* and *fri* backgrounds. Left to right: heart, torpedo and mature stage embryos, and seedlings at 1 day after germination. Scale bars: ovules, 20 μm; stamens, 100 μm; embryos, 100 μm; seeds, 100 μm.

maintained throughout germination (Figure 5b). Therefore, FRI might be dispensable for the reactivation of *FLC* in early embryogenesis, although it is required to maintain high levels of *FLC* expression in later embryonic and vegetative development. To further confirm the role of FRI in *FLC* reactivation during embryogenesis, we also analyzed the expression of *FLC::GUS* in the *suf4* mutant background. A loss of SUF4 activity has been reported to cause the decreased expression of *FLC*, as observed in *fri* plants, and the SUF4 protein has been reported to physically interact with FRI, and might recruit FRI to the *FLC* promoter (Kim *et al.*, 2006). Consistent with our results in *fri* plants, *FLC*

reactivation in early-stage embryogenesis was not affected by the *suf4* mutation (Figure 5a). In summary, our results from the *fri* and *suf4* plants demonstrate that FRI and SUF4 are not required for *FLC* reactivation, but are required for the maintenance of high levels of *FLC* expression in late embryogenesis and vegetative development.

Exchange of the histone variant H2A.Z with H2A has been proposed to play a critical role in epigenetic reprogramming in animals (Hajkova *et al.*, 2008). In Arabidopsis, a yeast SWR1-like PIE1-containing complex is involved in the H2A to H2A.Z exchange, and is required for the full activation of *FLC* in vegetative tissues (Deal *et al.*, 2007; Choi *et al.*, 2007; March-Diaz *et al.*, 2008). To test whether the PIE1 complex is also required for the reprogramming of *FLC*, *FLC::GUS* expression was studied in *pie1* mutants (Figure 5a). *FLC::GUS* was not expressed in the ovules and pollens of *pie1*: this might result from the suppression of *FLC* in diploid maternal tissues of *pie1*, as has been reported previously (Noh and Amasino, 2003). After fertilization, globular-stage embryos did not exhibit a detectable GUS signal (Figure S2e). Torpedo-stage embryos of the *pie1* mutant exhibited a weak GUS staining only in the basal region (Figure 5a). *FLC::GUS* was expressed strongly in root, but was expressed weakly in the shoot apex and vasculature of hypocotyl and cotyledons of fully matured *pie1* embryos. Therefore, these results indicate that the PIE1 complex is not relevant to the repression of *FLC* in gametophytes, but instead plays a pivotal role in the reactivation of *FLC* in early embryos, as well as in the maintenance of full activation of *FLC* in late embryos.

Polycomb group (PcG) complexes repress various sets of genes (Pien and Grossniklaus, 2007). In Arabidopsis, the vernalization-induced repression of *FLC* expression is maintained by the VRN2 PcG complex during vegetative development (Gendall *et al.*, 2001). Another PcG complex, the MEDEA (MEA)-Fertilization Independent Endosperm (FIE) PcG complex, acts in the endosperm, and represses genes that might cause endosperm overproliferation (Grossniklaus *et al.*, 1998; Kinoshita *et al.*, 1999; Kiyosue *et al.*, 1999; Kohler *et al.*, 2003, 2005; Gehring *et al.*, 2006). A recent study revealed that FIE also interacts with the VRN2 PcG complex, and is required for vernalization responses (Wood *et al.*, 2006). Hence, we hypothesized that the repression of *FLC* in the endosperm might be mediated by the MEA-FIE PcG complex. If the MEA-FIE PcG complex is responsible for the repression of *FLC* in the endosperm, we should be able to detect *FLC::GUS* expression in *mea* and *fie* mutant seeds. As discussed earlier, *FLC* is capable of being expressed in the endosperm, as shown by the *fve* mutant results in Figure 5a. Seeds of *mea* (Figure 6a,b) and *fie* mutants (Figure 6d,e) showed arrested embryos and an enlarged endosperm phenotype. However, the pattern of *FLC::GUS* expression was not altered by these PcG mutations during embryogenesis. We also examined *FLC::GUS* expression in emasculated *fie* mutant ovules (Figure 6c). We emasculated *fie* heterozygous flowers to determine whether *FLC::GUS* was derepressed in *fie* mutant ovules, because the *fie* mutation is embryonic lethal. As the *fie* mutation allows the division of diploid central cells without fertilization, we could easily distinguish *fie* mutant ovules from WT ovules in emasculated *fie* heterozygous plants. Again, we could not detect the derepression of *FLC::GUS* expression in the *fie* seed-like structures. We also examined the expression of *FLC* mRNA in the pollen of *fie* heterozygous plants, but were unable to detect expression by RT-PCR (Figure S3c). Therefore, the above data indicate that the repression of *FLC* in pollen, and in the endosperm of developing seeds, is not mediated by the MEA-FIE PcG complex, and should be regulated by a mechanism that is distinct from the one acting in vernalization in vegetative tissues.

Discussion

FLC *expression during gametogenesis*

Before fertilization, *FLC* expression was detected in ovules, but not in the pollen of non-vernalized plants (Figure 1a,b). Using a genetic test, we demonstrated that *FLC* expression

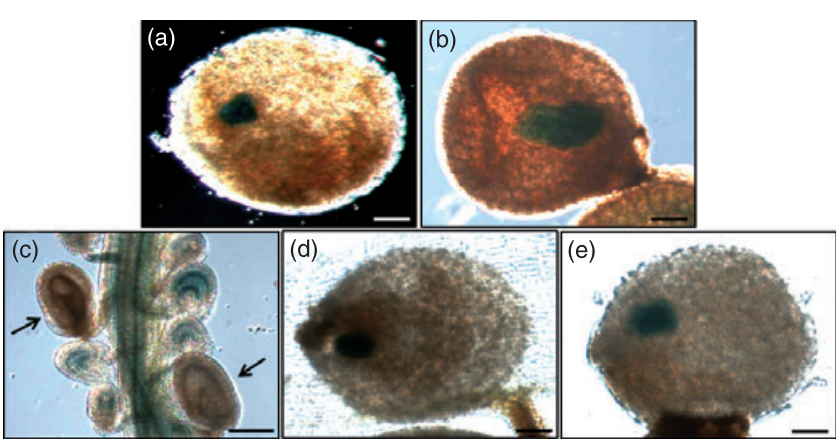


Figure 6. *FLC::GUS* expression in *mea* and *fie* mutants.
(a and b) *FLC::GUS* expression in *mea-3* mutant seeds. (a) Seven days after pollination (DAP); (b) 9 DAP; (c–e) *FLC::GUS* expression in *fie-1* heterozygous mutants. (c) Ovules at 6 days after emasculation. Seed-like structures containing 2n central-cell replication without embryos can be seen in *fie* mutant ovules (arrows); (d) 7 DAP; (e) 9 DAP. Scale bars: seeds, 100 μm; pistil, 50 μm.

in the ovule originates from the diploid maternal tissues that enclose female gametophytes (Figure S1). The expression pattern of *FLC* after vernalization treatment further supports this conclusion: when vernalization suppressed *FLC* expression, we could not detect any *FLC* expression in ovules (Figure 1c,d). Therefore, we conclude that *FLC* expression is fully repressed before gamete formation.

Recently, Sheldon *et al.* (2008) reported that *FLC* is reactivated temporarily in the developing somatic and sporogenous tissues of anthers, but is re-repressed in mature anthers. This temporary reactivation was observed using two independent *FLC::GUS* transgenic lines in either C24 or L*er* backgrounds. In this study, we did not observe the temporary reactivation of *FLC::GUS* throughout anther development in the Col-0 background (Figure 1 and data not shown). However, similar to the observation made by Sheldon *et al.* (2008), we observed *FLC::GUS* expression in the pollen sacs of the hybrid progeny of crosses between Col and L*er* plants (data not shown). The expression was restricted to somatic tissues such as tapeta, and was not observed in pollen grains. Therefore, the anther-specific temporary reactivation of *FLC* seems to vary depending on genetic background. In both studies, *FLC* expression is fully repressed in mature male gametophytes, as well as in female gametophytes.

Biological roles of autonomous-pathway genes in FLC *resetting*

The autonomous pathway represses *FLC* expression in the vegetative tissues of many summer-annual Arabidopsis accessions (Sheldon *et al.*, 1999, 2000; Michaels and Amasino, 2001). However, the repression of *FLC* by the autonomous pathway is fully suppressed by the transcriptional activating role of a functional *FRI* allele in winter-annual accessions (Johanson *et al.*, 2000). Because many summer-annual Arabidopsis accessions have arisen from loss-of-function mutations in *FRI* (Johanson *et al.*, 2000), the ancestral genetic composition of Arabidopsis should contain functional *FRI* alleles. Hence, the function of autonomous-pathway members in the vegetative tissues of *FRI*-containing ancestral or winter-annual genetic backgrounds is likely to balance the effects of FRI, to achieve a proper level of *FLC* expression. Because we observed the repression of *FLC* expression in gametophytes and the endosperm, we tested the possible repressive role of autonomous-pathway members on *FLC* in those reproductive tissues. We also observed that many of the autonomous-pathway genes are expressed in patterns similar to *FLC* in reproductive tissues, with a few exceptions (*HAC1*, *HAC5* and *HAC12*) that are also expressed at significant levels in pollen and the endosperm, where *FLC* is not expressed (Figure 4). However, our tests using several autonomous-pathway mutants revealed that none of these are involved in the repression of *FLC* in gametophytes and the endosperm (Figures S3 and 5), although we could not rule out the possibility that other autonomous-pathway genes, such as *FCA*, *FY*, *FPA*, *FLK* and *REF6*, that were not tested in this study might also repress *FLC*.

A recent study reported defective seed production in *fca fpa* double mutants (Baurle *et al.*, 2007), suggesting that FCA and FPA might also function in reproductive development. In this study, we show that the repression of *FLC* in the endosperm is mediated, at least in part, by FVE (Figures S2 and 5a). FVE was also reported to regulate cold responses (Kim *et al.*, 2004). Therefore, autonomous-pathway members might have multiple roles in various aspects of Arabidopsis development, as well as in *FLC* regulation, although the details of these roles have yet to be elucidated.

Roles of FRI *in the reactivation of* FLC *during embryogenesis*

When a dominant-active allele of *FRI* exists, *FLC* expression is activated even in the presence of functional autonomous-pathway repressors, and the plant shows a late-flowering phenotype. RT-PCR and the *GUS* fusion analyses in this study revealed that like *FLC* (Figure 1), *FRI* is expressed in the ovule and the embryo (Figure 3). However, the results presented in Figure 5 clearly show that an active *FRI* allele is dispensable for the initial reactivation of *FLC* in the embryo. We have also found that SUF4, an interacting partner of FRI (Kim *et al.*, 2006), has no role in *FLC* reactivation (Figure 5). These results, taken together, indicate that FRI and SUF4 are required for the activation of *FLC* after late embryogenesis, but not during early embryogenesis. Accordingly, *FLC* resetting in reproductive tissues should be initiated by a different mechanism from that which regulates *FLC* in vegetative tissues. It is possible that some of the factors isolated as *FLC* activators might be responsible for the initiation of *FLC* reactivation during early embryogenesis. To address this possibility, *FLC* expression must be analyzed during embryogenesis in mutant backgrounds of various *FLC* activators. Interestingly, the *FLC* transcript was not detected in *atx1-1* mutant embryos in a recent study (Pien *et al.*, 2008). Therefore, ARABIDOPSIS TRITHORAX 1 (ATX1) might be the factor required for *FLC* reactivation. ATX1 directly interacts with *FLC* chromatin, and is required for trimethylating H3K4 in the *FLC* locus of rapidly flowering accessions. These results indicate a FRI-independent function of ATX1 in *FLC* activation. Therefore, it is worthwhile to further address the functional relationship between *FLC* resetting and *ATX1*, or other *FLC* activators.

On the other hand, the high level of *FLC* expression in the early-stage embryos of *fri* plants was no longer maintained in late embryogenesis (Figure 5). As the expression of *FLC::GUS* remained high until late embryogenesis in autonomous-pathway mutants such as *fld* and *ld* (Figure 5), the repression of *FLC* expression in mature embryos of *fri* plants

might be mediated by autonomous-pathway genes. The fact that high *FLC::GUS* expression is maintained in the mature embryos of *FRI* plants (Figure 5) means that the hierarchy between autonomous-pathway genes and *FRI* also exists in late embryogenesis. Taken together, our results support the idea that *FRI* and autonomous-pathway genes determine the transcriptional activity of *FLC* during late embryogenesis, and this is important for the initial establishment of flowering competence.

Possible mechanisms for the reprogramming of FLC *during reproductive development*

Based on our tests of the role of *FLC* repressors and the MEA-FIE PcG complex in silencing *FLC* in gametophytes, none of these repressors or the components of the PcG complex were responsible for the repression of *FLC* in gametophytes (Figures 5 and 6). Although we cannot exclude the possibility of activity by other *FLC* repressors not tested in this study, it is possible that the silencing of *FLC* in gametophytes is established by the canonical process of epigenetic reprogramming, rather than by specific *FLC* repressors.

A dynamic exchange of histone proteins in mouse germ cells was reported recently (Hajkova *et al.*, 2008). The authors demonstrated that the dynamic exchange of histone H2A with its variant H2A.Z occurs before the production of totipotent germ cells, and suggested the importance of this exchange in the erasure of epigenetic modifications that are pivotal for genomic reprogramming. In Arabidopsis, a yeast SWR1-like PIE1-containing complex mediates the exchange of H2A with H2A.Z at the *FLC* locus in vegetative tissues (Choi *et al.*, 2007). Our results in Figure 5a demonstrate that the PIE1 complex is not required for the repression of *FLC* in gametophytes, but is essential for the reactivation and maintenance of *FLC* expression in early and late embryogenesis, respectively. Therefore, the H2A to H2A.Z exchange is likely to play a critical role in epigenetic reprogramming in Arabidopsis.

Changes in genome-wide DNA methylation are involved in genomic reprogramming in mammals (Reik *et al.*, 2001). Interestingly, *FLC* transcription is low in the vegetative tissues of hypomethylated Arabidopsis mutants, such as *ddm1* or *antisense MET1* transgenic plants (Jean Finnegan *et al.*, 2005). Although changes in *FLC* expression in both mutants were suggested as an indirect effect of the changes

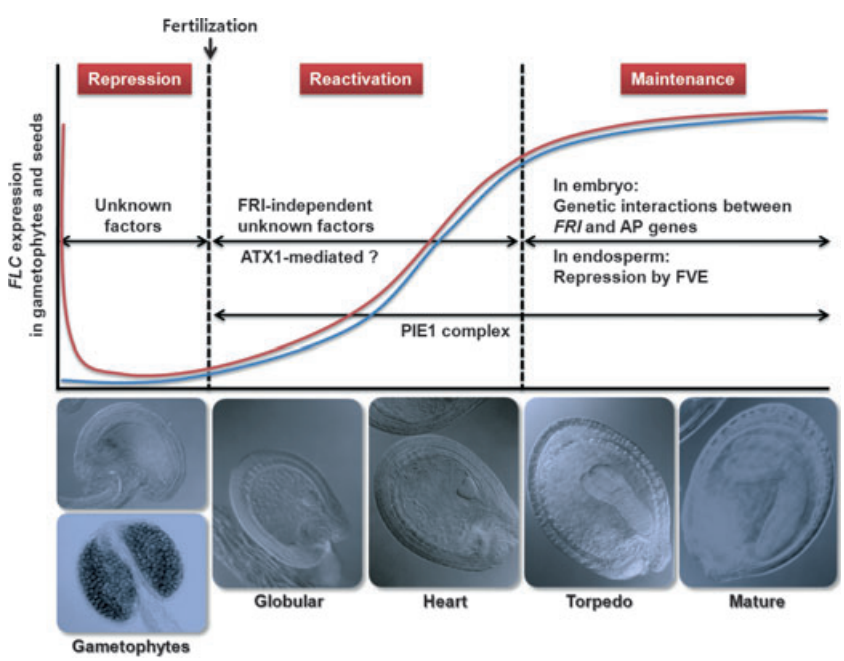


Figure 7. Schematic model for *FLC* reprogramming during reproductive development.
The process of *FLC* reprogramming during reproductive development might be divided into three phases: (i) a phase of repression in gametogenesis, (ii) a phase of reactivation in early embryogenesis, and (iii) a phase of maintenance in late embryogenesis. Regardless of the epigenetic state in adult plants, *FLC* expression is repressed in male and female gametophytes by unknown mechanisms. Then, *FLC* expression is reactivated after fertilization in embryos, but not in the endosperm. The present study demonstrates that the reactivation process is independent of *FRI* and *SUF4*, but requires *PIE1* function. Although Pien *et al.* (2008) reported the failure of *FLC* reactivation in early-globular stage embryos of *atx1* mutants through an *in situ* hybridization study, the role of *ATX1* in *FLC* reactivation may need to be re-evaluated in an *FRI*-containing genetic background. At stages around maturation, the level of *FLC* expression in embryos is determined by genetic interactions between *FRI* and autonomous pathway (AP) genes. PIE1 is also necessary for the full activation of *FLC* at these stages, as well as for the initial reactivation. Interestingly, the repression of *FLC* in endosperm is mediated, at least in part, by an autonomous pathway gene *FVE*. The *FLC* expression in non-vernalized (red line) or vernalized (blue line) *FRI*-containing WT plants is depicted. Maternally transmitted *FLC* expression in ovules or early stage seeds is not considered in this model, to avoid misconception.

in genomic DNA methylation, the relationship between *FLC* resetting and DNA methylation during reproductive development has yet to be determined. In this study, we have addressed the reprogramming of *FLC* and the expression patterns of a number of its regulators during gametogenesis and embryogenesis. The model in Figure 7 summarizes the three phases of *FLC* reprogramming, and the role of some *FLC* regulators during the process, as revealed from our study. These results provide new insights into *FLC* reprogramming and the mechanisms for epigenetic reprogramming in general, in flowering plants.

Experimental procedures

Plant materials and growth conditions

All plants used in this study are in the Col-0 background, except for *vrn1*, *vrn2*, *mea-3* and *fie-1* (L*er* background) or *pie1-1* (Ws background). Seeds were stratified on 0.65% phytoagar containing half-strength MS (Plantmedia, http://www.plantmedia.com) salts for 3 days at 4°C. All plants were grown in long-day photoperiodic conditions (16-h light/8-h dark) under cool, white fluorescence light (100 μmole m^{-2} s^{-1}) at 22°C, with 60% relative humidity. For vernalization, seedlings germinated on MS plates were incubated for 4 weeks at 4°C under short-day conditions (8-h light/16-h dark). Afterwards, vernalized seedlings were further grown on soil with long days.

Generation of FRI::GUS, VRN1$_{pro}$::VRN1:GUS *and* VRN2$_{pro}$::VRN2:GUS *plants*

The procedures used to generate these *GUS* fusion constructs and transgenic plants are described in Appendix S1.

Histochemical GUS imaging

The GUS activity in gametophytes and seeds was analyzed by incubation in 50 mM $NaPO_4$ (pH 7.0), 1 mM X-Gluc (Sigma-Aldrich, http://www.sigmaaldrich.com), 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 10 mM EDTA and 0.2% Triton X-100 at 37°C for 8–10 h. After staining, tissues were cleared by incubation in 70% EtOH for several hours. Stained tissues were photographed using an Axio Imager A1 microscope (Carl Zeiss, http://www.zeiss.com), with an AxioCam HRc camera.

Analysis of gene expression

Methods for pollen collection were described previously by Choi *et al.* (2002). To analyze gene expression in ovules, pistils were dissected before fertilization, and exposed ovules and placentas were harvested for total RNA extraction. Total RNA extraction from developing seeds was performed using seeds independently harvested according to the stage of embryo development.

Total RNA from various vegetative tissues and gametophytes were extracted with Trizol reagent (Invitrogen, http://www.invitrogen.com) following the manufacturer's protocol. For RNA extraction from seeds, the Ambion RNAqueous™ Kit (Ambion, http://www.ambion.com) was used. A total of 1–2 μg of RNA was reverse transcribed using M-MLV reverse transcriptase (Ambion) and an oligo-dT primer after RNase-free DNase treatment (TaKaRa Bio, http://www.takara-bio.com). PCR amplification was performed using gene-specific primers (see Table S1 for primers).

Real-time qPCR

Real-time qPCR was performed on 96-well optical reaction plates (Bio-Rad, http://www.bio-rad.com). All PCR mixtures contained 10 μl of iQ™ SYBR green Supermix (Bio-Rad), 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer (10 μM) and 5 μl of each diluted RT product per well. PCR amplification of the *TUB2* housekeeping gene was performed as a control for sample loading, and for normalization. Negative controls were treated the same way as the samples, but without reverse transcriptase. All of the templates were run in triplicate, and the threshold cycle (C_t) was determined using iQ™ OPTICAL SYSTEM Software (Bio-Rad). Gene-specific transcripts were quantified using the ddC_t method (ddC_t = $C_{t\ gene\ of\ interest}$ − $C_{t\ TUB2}$). Real-time SYBR-green dissociation curves showed one species of amplicon for each primer combination.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *FLC::GUS* expression in ovules of *FLC::GUS* hemizygous plants.

Figure S2. *FLC::GUS* expression in *fve* and *pie1* mutants.

Figure S3. *FLC* expression in *hac* and *fie* mutants.

Table S1. Primers used for RT-PCR analysis.

Appendix S1. Supplementary experimental procedures

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