Resetting of *FLOWERING LOCUS C* expression after epigenetic repression by vernalization

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The epigenetic repression of *FLOWERING LOCUS C* (*FLC*) in winter-annual ecotypes of *Arabidopsis* by prolonged cold ensures that plants flower in spring and not during winter. Resetting of the *FLC* expression level in progeny is an important step in the life cycle of the plant. We show that both the paternally derived and the maternally derived *FLC*:*GUS* genes are reset to activity but that the timing of their first expression differs. The paternal *FLC*:*GUS* gene in vernalized plants is expressed in the male reproductive organs, the anthers, in both somatic tissue and in the sporogenous pollen mother cells, but there is no expression in mature pollen. In the progeny generation, the paternally derived *FLC*:*GUS* gene is expressed in the single-celled zygote (fertilized egg cell) and through embryo development, but not in the fertilized central cell, which generates the endosperm of the progeny seed. *FLC*:*GUS* is not expressed during female gametogenesis, with the maternally derived *FLC*:*GUS* being first expressed in the early multicellular embryo. We show that *FLC* activity during late embryo development is a prerequisite for the repressive action of *FLC* on flowering.

Arabidopsis | embryo | gametogenesis

Flowering of many plants is induced by the environmental cues photoperiod and prolonged low temperature (vernalization). With photoperiod control, flowering is induced in response to a critical day length, either long or short days depending on the species. In *Arabidopsis*, a long-day plant, the gene *CONSTANS* (*CO*) is triggered by long days to activate *FLOWERING LOCUS T* (*FT*) and is antagonistic to the action of the FLOWERING LOCUS C (FLC) protein (1), which binds to *FT* to repress its activity and prevent flowering (2, 3). *FLC* is repressed by vernalization (4, 5), so that after the cold exposure *FLC* activity is low, releasing the repression of *FT* and allowing CO-mediated activation of *FT* in the long days of spring. The repression of *FLC* by vernalization occurs because of changes in *FLC* chromatin. The low-temperature exposure induces expression of VERNALIZATION INSENSITIVE3 (6), which interacts with the VERNALIZATION2 polycomb-like complex (7). This complex binds to *FLC* chromatin (6), modifying histone residues, including the trimethylation of lysine-27 of histone H3, and expression is repressed (6, 8, 9).

The chromatin control of *FLC* activity is an example of epigenetic control of gene expression. The repressed state is retained through successive mitotic divisions throughout the development of the plant after the period of low temperature ends, and the gene is then reset to an active transcriptional state in the next sexual generation (10). This mode of control of *FLC* activity ensures that *FT* is repressed before winter so that the long-day photoperiod of spring is able to induce *FT* activity, with flowering occurring at an optimal time.

Nothing is known about the mechanism of resetting *FLC* gene activity or of the timing of this event. In this article we show that the activities of both paternally and maternally derived *FLC*:*GUS* reporter genes are reset after vernalization, but the timing of their initial expression differs. The paternal gene copy is active during early gametogenesis and in the single-celled zygote, whereas the maternal copy is not expressed until the early multicellular embryo stage.

Results

FLC:_GUS_ Is Expressed in the Somatic and Sporogenous Tissues of Anthers After Vernalization. In nonvernalized C24 ecotype plants, which carry an active *FRIGIDA* (*FRI*) allele, *FLC* and a reporter construct with the C24 *FLC* allele linked to the GUS coding region (*FLC-C24*:*GUS*) were expressed in the vegetative plant, and their expression was repressed in vernalized plants [supporting information (SI) Fig. 6] (4). After vernalization, repression of *FLC* and *FLC-C24*:*GUS* is maintained during and after the transition to flowering, but activity is reset in progeny plants (SI Fig. 6) (10). To determine when resetting occurs, we have used the *FLC-C24*:*GUS* reporter to follow activity during gametogenesis and embryogenesis.

In flower buds from nonvernalized plants, *FLC-C24*:*GUS* was expressed in both the male and female reproductive structures, the anthers and carpels (Fig. 1*A*), whereas expression in vernalized flower buds was restricted to anthers (Fig. 1 *B* and *E*). The behavior of the reporter gene is consistent with the expression pattern of the endogenous *FLC* gene (Fig. 1*C*).

In vernalized plants, there was no *FLC-C24*:*GUS* activity in stage-8 flower buds (flower stages from ref. 11) that contained stage-4 anthers (anther stages from ref. 12) (Fig. 1 *D* and *F*) or in earlier bud stages. GUS activity was first detected in stage-5 anthers and was present in pollen mother cells (PMC), in the tapetum surrounding the PMC, in other cell layers of the anther wall, and in the anther connective tissue (Fig. 1 *E* and *G*). GUS activity was also present within PMC undergoing meiosis in stage-6 anthers (Fig. 1*H*) and at the tetrad stage in stage-7 anthers (Fig. 1*I*). In stage-8 anthers, there was a low level of GUS activity in the haploid microspores released from the tetrads and in other tissues (Fig. 1*J*). Before and during deposition of the pollen coat, there was a high level of *FLC*:*GUS* activity in the tapetum (Fig. 1 *J* and *K*). *FLC*:*GUS* activity decreased markedly as the tapetum degenerated in stage-10 to -11 anthers, but occasional GUS crystals were still evident in mitotic pollen grains (Fig. 1*L*). No GUS activity was detectable in tricellular pollen (Fig. 1*M*). A similar pattern of expression was obtained with a second *FLC*:*GUS* construct that contained the Columbia (Col) *FLC* sequence in Landsberg *erecta* carrying the active *FRI*-H51 allele (*FLC-Col*:*GUS* in L*er FRI*). These observations indicate that *FLC*:*GUS* activity has been reset in the male reproductive structure in both sporogenous and somatic tissues and that the gene becomes inactive in maturing pollen.

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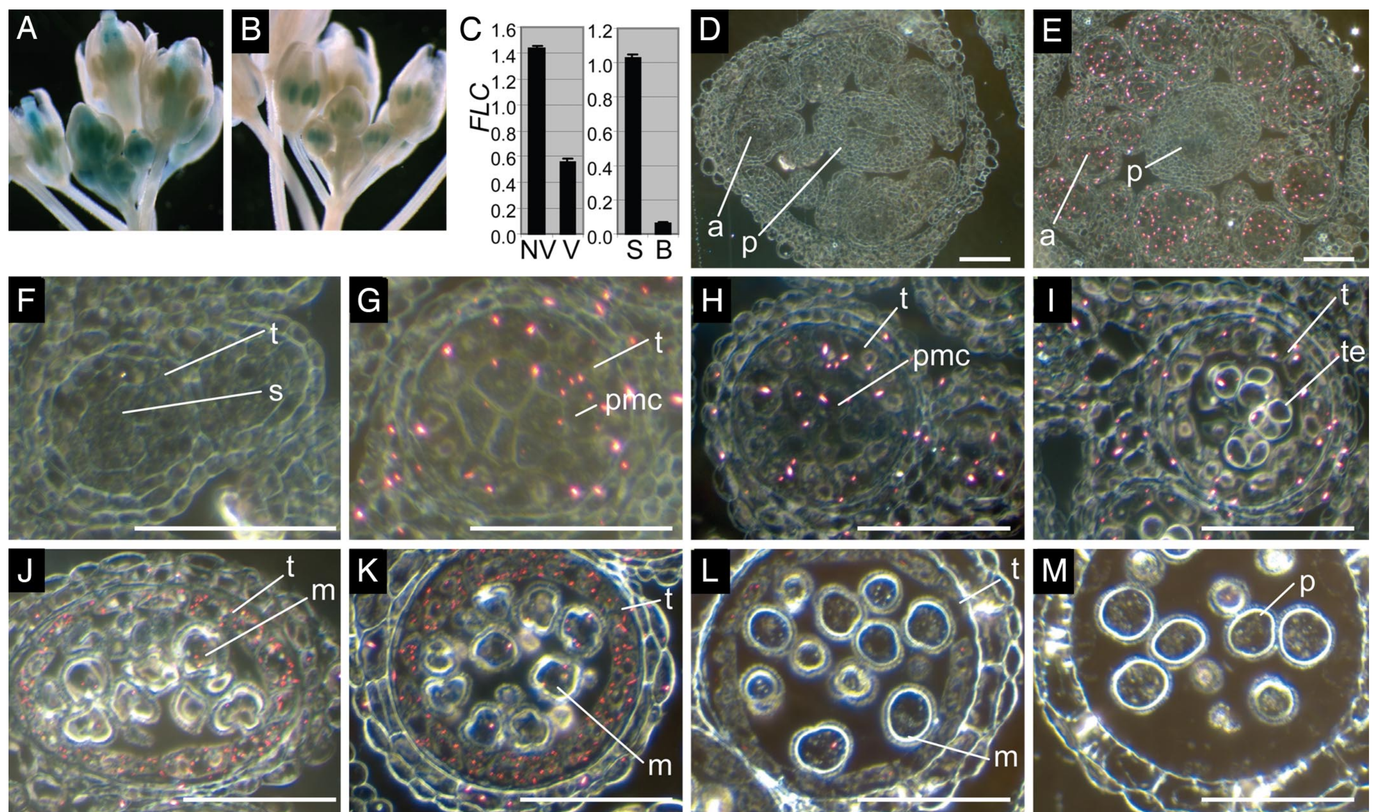

Fig. 1. *FLC-C24*:*GUS* is reset in the somatic and sporogenous tissues in the anther. (*A* and *B*) GUS-stained buds from nonvernalized (*A*) and vernalized (*B*) C24 + *FLC-C24*:*GUS* plants. (*C*) Quantitative RT-PCR comparing *FLC* expression in nonvernalized (NV) and vernalized (V) unopened buds and comparing expression in stamens from young buds (S) and remaining bud tissue (B), indicating that expression in young vernalized buds is largely limited to stamens. (*D*–*M*) Transverse sections through consecutive buds from vernalized C24 + *FLC-C24*:*GUS* plants viewed under dark-field conditions in which the GUS crystals appear pink (*F* and *G* are higher-magnification images of *D* and *E*, respectively). (*D* and *F*) Flower bud at late stage 8, anther stage 4 with sporogenous cells. (*E* and *G*) Flower bud at early stage 9, anthers at stage 5 with premeiotic or early meiotic PMC. (*H*) Stage-6 anther with PMC in meiosis. (*I*) Stage-7 anther containing tetrads. (*J* and *K*) Stage-8 anther with haploid microspores showing induction of expression in tapetum. (*L*) Stage-10 to -11 anther with degenerating tapetum. (*M*) Stage-12 anther. The tapetum has degenerated, and two pollen mitoses are complete. a, anther; m, microspore; p, pistil; s, sporogenous cells; t, tapetum; te, tetrad. (Scale bars: 50 μm.) Flower stages are from ref. 11, and anther stages are from ref. 12.

***FLC*:*GUS* Is Expressed in the Next Generation Zygote.** In contrast to the resetting of *FLC*:*GUS* that occurs during the development of the male reproductive structures, there was no detectable expression of either *FLC-C24*:*GUS* or *FLC-Col*:*GUS* during female meiosis, after formation of the functional megaspore, or in the mature female gametophyte of ovules on vernalized plants (Figs. 2 *B*, *E*, *G*, and *J* and 3*D*). Nonvernalized ovules expressed *FLC*:*GUS* strongly in the integuments (Fig. 2 *A* and *D*).

Approximately 24 h after pollination, weak *FLC-Col*:*GUS* activity was detected in the single-celled zygote formed by the fusion of the egg cell with a male gamete (Fig. 2 *C* and *F*). There was no GUS activity in the endosperm, the product of fertilization of the diploid central cell by a second male gamete, or in the integuments. Expression in the single-celled zygote was rarely detectable in the C24 + *FLC-C24*:*GUS* line, which had an overall lower level of expression than the L*er FRI* + *FLC-Col*:*GUS* line.

The Timing of Expression Is Different for Maternal and Paternal *FLC*:*GUS* Genes. We investigated whether both the male- and female-derived *FLC-Col*:*GUS* genes were expressed in the zygote. When the vernalized *FLC-Col*:*GUS* gene copy was contributed by the male, activity was observed in single-cell zygotes (Fig. 2*H*) at the same stage as in selfed vernalized plants (Fig. 2*C*). The frequency of detectable GUS activity of the male-derived *FLC*:*GUS* gene was lower (10–20%) than that of zygotes generated by self-pollination of a homozygous *FLC*:*GUS* plant (50%). The reason for this difference is not known. Expression of the male-derived *FLC*:*GUS* gene continued in early-globular and subsequent-stage embryos (Fig. 2*K*). When the vernalized *FLC-Col*:*GUS* gene copy was contributed by the female, no expression was seen in the zygote and the earliest expression was around the early-globular stage (Fig. 2 *I* and *L*).

Endogenous *FLC* Expression Affects Early Embryo Expression of *FLC*:*GUS*. When pollen containing *FLC-C24*:*GUS* from either a nonvernalized *flc*-null (*flc-20*) or *FLC* wild-type (C24) plant was used to fertilize nonvernalized *flc-20* or C24 plants, GUS activity was reduced when both parents contributed wild-type *FLC* alleles compared with when either parent, or both, contributed an *flc-20* allele (Table 1). This suggests that endogenous *FLC* expression, from either the maternally or paternally derived gene copy, directly or indirectly results in decreased expression of the male-derived *FLC-C24*:*GUS* gene in early-globular embryos developing in ovules of nonvernalized plants. This effect is short-lived, because by the late-globular stage the male-derived *FLC(C24)*:*GUS* embryonic expression was similar regardless of the *FLC* genotype (data not shown).

Early-globular embryos from vernalized C24 plants had increased expression of the male-derived *FLC-C24*:*GUS* transgene compared with embryos from nonvernalized C24 (Table 1). This observation suggests that early embryos developing on vernalized plants have a reduced level of endogenous *FLC* expression compared with embryos developing on a nonvernalized plant,

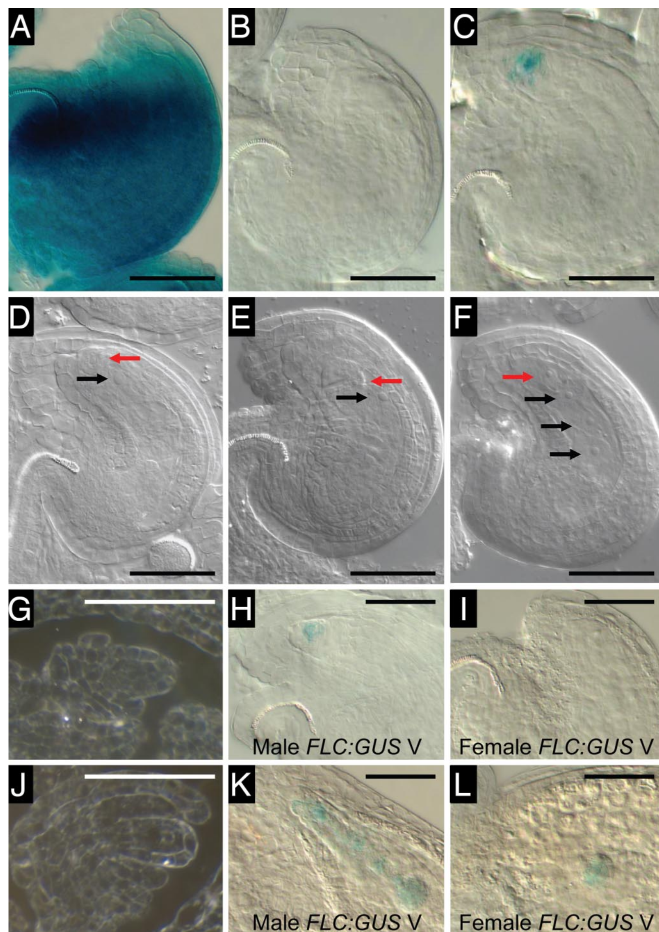

Fig. 2. *FLC*:*GUS* is not expressed during female gametogenesis, and the paternally derived gene is expressed in the single-celled zygotes from vernalized plants. (*A* and *D*) Youngest open pollinated flower of nonvernalized L*er FRI* + *FLC-Col*:*GUS*. (*B* and *E*) Youngest open pollinated flower of vernalized L*er FRI* + *FLC-Col*:*GUS*. (*C* and *F*) Second youngest open pollinated flower of vernalized L*er FRI* + *FLC-Col*:*GUS*. (*A*–*C*) GUS-stained viewed with DIC optics. (*D*–*F*) Cleared ovules for stage comparison. (*G* and *J*) Sections of C24 + *FLC-C24*:*GUS* through ovule primordia around the time of meiosis (*G*) and at the functional megaspore stage (*J*) viewed under dark-field conditions. (*H* and *K*) Ovules resulting from a cross between male L*er FRI* + *FLC-Col*:*GUS* vernalized and female L*er FRI*-Sf2 vernalized. (*I* and *L*) Ovules resulting from a cross between female L*er FRI* + *FLC-Col*:*GUS* vernalized and male L*er FRI*-Sf2 vernalized. (*H* and *I*) Single-celled zygote stage ovules (1 day after pollination). (*K* and *L*) Early-globular stage embryos (3 days after pollination). Black arrows, endosperm nuclei; red arrows, zygotic nuclei. (Scale bars: 50 μm.)

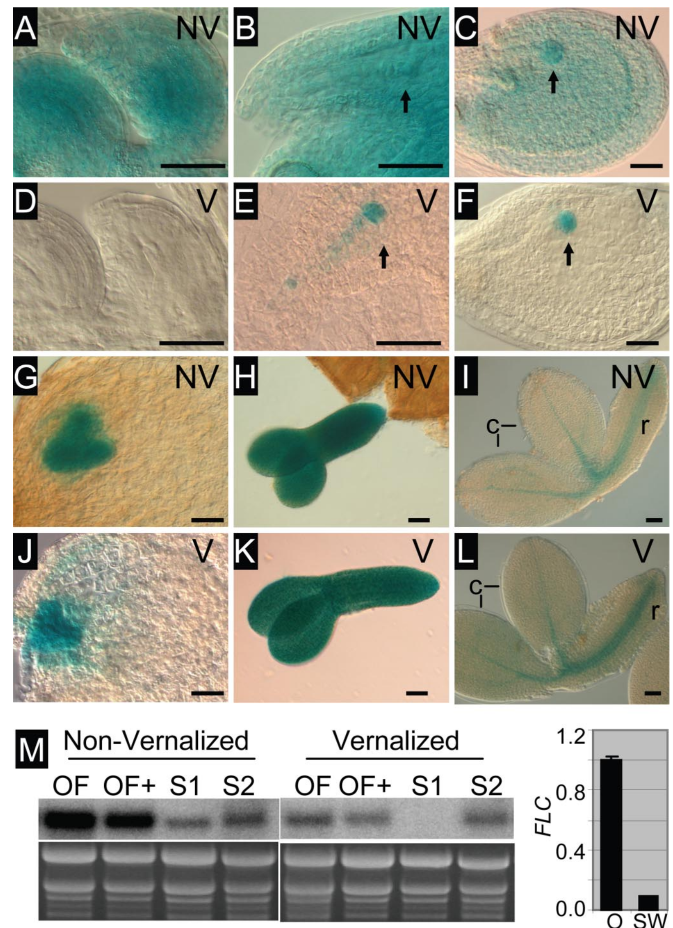

Fig. 3. *FLC-C24*:*GUS* is expressed throughout embryo development. (*A*–*L*) GUS-stained ovules and embryos from nonvernalized (NV) or vernalized (V) C24 + *FLC-C24*:*GUS* plants. (*A* and *D*) Unfertilized ovules. (*B* and *E*) Ovules with single-cell pro-embryo and suspensor indicated by arrow. (*C* and *F*) Ovules with globular embryo indicated by arrow. (*G* and *J*) Ovules with heart-stage embryo. (*H* and *K*) Torpedo-stage embryos. (*I* and *L*) Late-stage (bent cotyledon) embryos. (*M Left*) RNA gel blot showing *FLC* expression in flowers and siliques from nonvernalized and vernalized C24 plants. OF, open flowers; OF+, open flowers with up to 5-mm siliques (containing pro-embryos up to the four-cell stage); S1, siliques between 5 and 10 mm in length (containing embryos from four-cell to globular stage); S2, siliques >10 mm (containing embryos larger than globular stage). The ethidium bromide-stained gel is shown as a loading control. (*M Right*) Quantitative RT-PCR comparing *FLC* expression in ovules (O) and silique walls (SW) from ≈10-mm siliques from vernalized plants.

consistent with the maternally derived *FLC*:*GUS* gene being first expressed around the early-globular stage (Fig. 2 *I* and *L*).

FLC:GUS Is Expressed Throughout Embryogenesis. In nonvernalized flowers *FLC-C24*:*GUS* activity was present in the ovule integuments before and after pollination and in the pro-embryo and globular-stage embryo 2–3 days after pollination (Fig. 3 *A*–*C*). Around the late-globular to heart stage of embryo development only embryo expression and not integument expression is evident (Fig. 3*G*). At early to mid stages of embryo development GUS activity was present throughout the embryo (Fig. 3 *C*, *G*, and *H*). In older embryos, GUS activity was strongest in the provascular tissue of both the embryonic root and cotyledon (Fig. 3*I*).

In fertilized vernalized ovules, *FLC-C24*:*GUS* activity was present in the single-celled embryo and attached suspensor and in globular embryos, but not in the endosperm or in the integuments (Fig. 3 *E* and *F*). At the heart stage of embryo development and subsequently, GUS activity was identical in nonvernalized and vernalized ovules (Fig. 3 *G*–*L*).

FLC(C24):*GUS* activity is consistent with the expression of the endogenous *FLC* gene (Fig. 3*M*). In flowers containing ovules at the zygote stage, at the one- to four-cell pro-embryo stage, and in siliques containing ovules at the four-cell to globular embryo stage, there is higher expression in the nonvernalized compared with the vernalized samples, consistent with repression of maternal expression in the vernalized samples. In siliques containing larger embryos, expression of the nonvernalized and vernalized samples was similar, consistent with *FLC* expression having been reset by this stage. Expression in vernalized siliques is largely limited to ovules (Fig. 3*M*).

FLC Expression During Late Embryogenesis Is Required for Late Flowering. To address the role of *FLC* expression during embryo development, we used a bipartite dexamethasone inducible

Table 1. Paternally derived *FLC-C24:GUS* activity in early-globular embryos is reduced by endogenous *FLC* expression

Female parent	Male parent*	Embryo *FLC* genotype	No. of embryos† (%)		
			N	S	Total
C24 NV	C24 NV	*FLC/FLC*	173 (86.1)	28 (13.9)	201
flc-20 NV	C24 NV	*flc/FLC*	84 (59.2)	58 (40.8)	142
C24 NV	*flc-20* NV	*FLC/flc*	27 (57.4)	20 (42.6)	47
flc-20 NV	*flc-20* NV	*flc/flc*	37 (55.2)	30 (44.8)	67
C24 V	C24 V	*FLC(reset)/FLC(reset)*	90 (62.5)	54 (37.5)	144

*Also carries *FLC-C24:GUS*.
†N, no detectable staining; S, detectable staining. Similar results were obtained in independent experiments.

system (13). In this line (*LhGR/pOp-FLC/GUS* in L*er*) both the *FLC* cDNA and *GUS* gene are induced by application of dexamethasone, with expression under the control of the 35S promoter that drives the activator construct (*35S:LhGR*). The level of FLC protein expression in induced vegetative plants was similar to that of 35S:FLC vegetative plants (data not shown). In the absence of dexamethasone no induction of either *FLC* or *GUS* occurred (Fig. 4 *I* and *J* and data not shown). Seeds were harvested from plants grown either with or without dexamethasone during flower and seed formation and were subsequently grown either with or without dexamethasone treatment from imbibition. All dexamethasone-treated and control plants flowered at around the same time as wild-type L*er* (Fig. 4*K*). L*er* + *35S:FLC* plants are late-flowering (4), suggesting that dexamethasone did not induce *FLC* in the same tissues as *35S:FLC*.

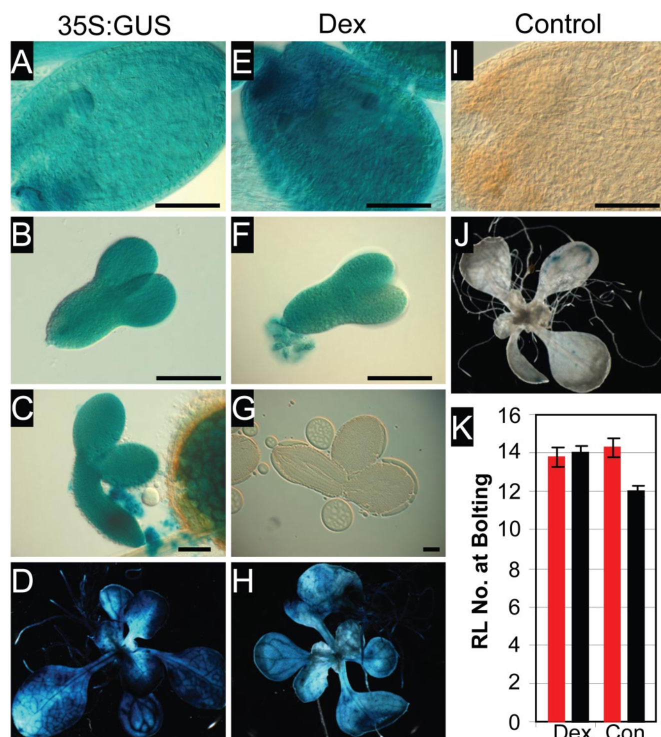

Fig. 4. *FLC* expression in late embryogenesis is required for delayed flowering. (*A–D*) 35S:GUS as a marker for 35S:FLC. (*E–H*) Dexamethasone-treated *LhGR/pOp-FLC/GUS* in L*er*. (*I* and *J*) Control-treated *LhGR/pOp-FLC/GUS* in L*er*. (Scale bars: 100 μm.) (*K*) Flowering time measurements of *LhGR/pOp-FLC/GUS* in L*er*, either dexamethasone-treated (Dex) or control-treated (Con). Seeds for the flowering time experiment came from plants that were either dexamethasone-treated (red bars) or control-treated (black bars) throughout vegetative growth, flowering, and seed development. Error bars indicate the standard error.

Dexamethasone induced both *GUS* and *FLC* expression during the vegetative phase of growth (Fig. 4*H* and data not shown), and GUS expression was detectable during early to mid embryogenesis (Fig. 4 *E* and *F*). After the torpedo stage of embryo development, dexamethasone appeared not to penetrate the developing seed coat because embryos did not express GUS (Fig. 4*G*). *35S:GUS* (as a marker for *35S:FLC*), in contrast, was expressed throughout the vegetative phase as well as throughout embryogenesis (Fig. 4 *A–D*). Expression of *FLC* during early to mid embryogenesis and during vegetative growth was not sufficient to confer late flowering in the dexamethasone-induced line, suggesting that expression of *FLC* during late embryogenesis, as well as during vegetative growth, is required for the plant to be late-flowering.

We used a related bipartite expression system (13) in which the reporter construct, containing both the *FLC* cDNA and *GUS* gene (*pOp-FLC/GUS*), was activated by a range of promoters, with differing tissue specificity, used in the activator constructs (*promoter:LhG4*). F_1 progeny resulting from crossing of the reporter line and the activator lines all displayed a delay in flowering (Fig. 5*A*). *CLAVATA* (*CLV*):*LhG4* × *pOp-FLC/GUS* strongly delayed flowering and directed expression in embryos from heart stage onwards, including in the provascular tissue of the cotyledons, as well as strong expression in the vegetative plant (Fig. 5 *A* and *E–G*). *SHOOTMERISTEMLESS* (*STM*):*LhG4* × *LhGh/pOp-FLC/GUS*, in contrast, although expressed strongly at the heart stage, had weak expression in the shoot apical meristem region of late-stage embryos and in vegetative plants and had a weak effect on flowering (Fig. 5 *A* and *N–P*). *SUCROSE TRANSPORTER2* (*SUC2*):*LhG4* × *LhGh/pOp-FLC/GUS*, which conferred a substantial delay on flowering, was not expressed in early-stage ovules but was expressed in late-stage embryos including in the provascular tissue of the root and cotyledon and in the phloem of vegetative plants (Fig. 5 *A* and *K–M*). *AINTEGUMENTA* (*ANT*):*LhG4* × *LhGh/pOp-FLC/GUS* and *CUP-SHAPED COTYLEDONS2* (*CUC2*):*LhG4 x LhGh/pOp-FLC/GUS* were both expressed in heart-stage embryos and also in the provascular tissue of late-stage embryos, as well as in the vegetative plant, and both conferred a moderate delay on flowering (Fig. 5 *A–D* and *H–J*). *FLC-C24:GUS* was expressed in the provascular cells of late-stage embryos (Fig. 3 *I* and *L*). *FLC* has been shown to function in the leaves of vegetative plants to repress *FT* expression in the companion cells of the phloem (3). *FT* is also expressed in late-stage developing seeds (14). The correlation between expression of *FLC* in the provascular cells of late-stage embryos under the control of a range of promoters and the ability to delay flowering suggests that FLC activity may be required to repress *FT* expression in the provascular cells in late-stage embryos, thereby preventing precocious flowering.

Discussion

The resetting of vernalization-repressed *FLC* in the next sexual generation ensures the need for vernalization in each generation

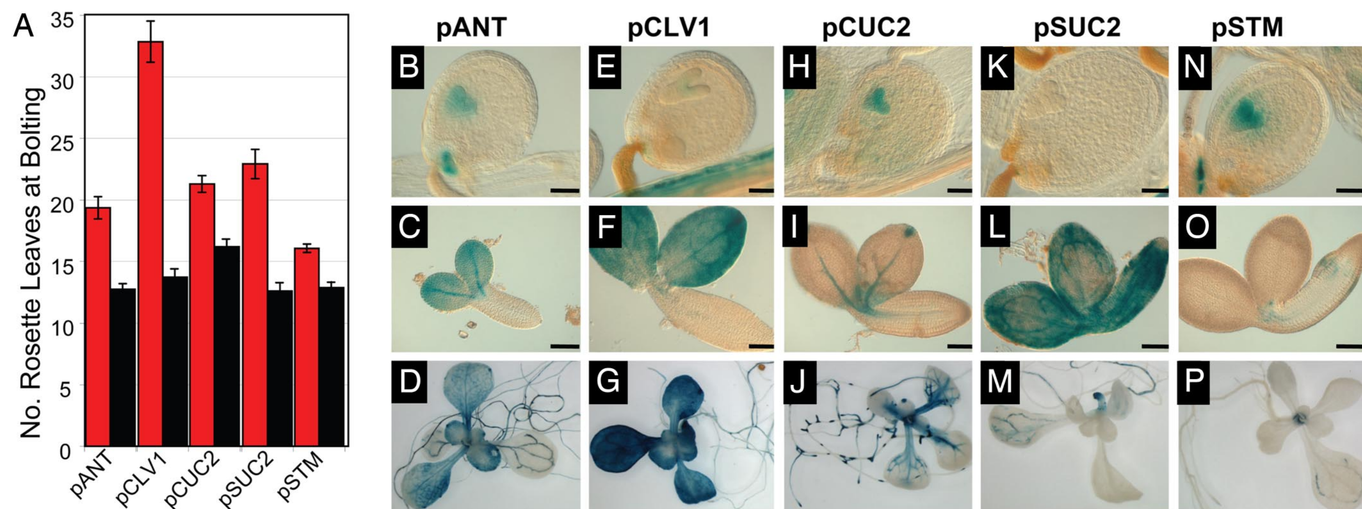

Fig. 5. *FLC* expression under the control of heterologous promoters results in delayed flowering. (*A*) Flowering time measurement of F_1 plants of *promoter:LhG4* × *LhGh/pOp-FLC/GUS* in L*er* (red bars) and the corresponding *promoter:LhG4* line in L*er* (black bars), with the promoter indicated on the *x* axis. The standard errors are indicated. (*B*, *E*, *H*, *K*, and *N*) Ovules containing heart-stage embryos. (*C*, *F*, *I*, *L*, and *O*) Dissected bent-cotyledon-stage embryos. (*D*, *G*, *J*, *M*, and *P*) Ten-day-old vegetative plants. The promoter driving *FLC* and *GUS* expression is indicated at the top. (Scale bars: 100 μm.)

to promote flowering. This study determines the timing of the first *FLC*:*GUS* expression in the progeny generation. We have used a functionally active region of the *FLC* gene (SI Fig. 6) (15) linked to the GUS reporter gene to follow resetting of *FLC*. This construct appears to have the same pattern of activity and vernalization response as the endogenous gene, but by following the activity of the GUS enzyme we are limited to observations on translational expression as opposed to transcriptional expression. The mitotic transmission of the repressed *FLC* state in the vernalized generation is associated with changes in *FLC* chromatin structure, including repressive histone modifications. Resetting presumably involves removal of these repressive histone modifications; however, this may not be sufficient for expression if required transcription factors are not present. Our analysis describes the timing of FLC:GUS expression but does not exclude the possibility of earlier events that result in an expression-competent *FLC* gene state.

In anthers of vernalized plants, *FLC*:*GUS* expression occurred in PMC and in somatic tissues of the anther, including the tapetum. The expression in PMC commenced before meiosis or in the early stages of meiosis and continued at a low level in the meiotic cells and in haploid microspores but ceased before pollen maturation. Although expression is observed around the time of meiosis, expression is not restricted to cells that undergo meiosis, indicating that meiosis is not required for resetting. *FLC*:*GUS* expression in the tapetum is initially low but increases markedly after release of the haploid microspores from the tetrads. During this stage the tapetal cells become binucleate and secrete materials for the formation of the pollen coat (16). *FLC*:*GUS* expression in the tapetum ceased as the tapetum degenerated before maturity of the anther. The observation of tapetum expression is consistent with the report of Zhang *et al.* (17) that *FLC* is high in nonvernalized wild-type young anthers and that expression is significantly reduced in mutant anthers that lack tapetal tissue. The function of *FLC* in these cells is not known. Plants without a functional *FLC* gene are fully fertile and have normal anther development. This may indicate that *FLC* function, if normally required, can be replaced by the action of other genes, perhaps the *FLC*-like *MAF* genes (18, 19).

Expression of *FLC*:*GUS* in a range of tissue types of the anther argues against resetting occurring in a common progenitor cell. The sporogenous cells and three cell layers of the anther wall, the tapetum, the middle layer, and the endothecium, have a common cellular origin, with a single archaesporial cell in the L2 layer of the stamen primordium giving rise to all four cell types. However, the other tissue types in which *FLC*:*GUS* is reset, the epidermis and the connective tissue, originate from L1 and L3, respectively (16).

The loss of *FLC*:*GUS* expression in tricellular pollen may be related to a general reduction in transcriptional activity as the chromatin of the gametes becomes compacted (20). Histone H3 variants and other histone variants have been reported in the two products of the first pollen mitosis, the generative cell and vegetative cell (21, 22), and it is possible that these variants contribute to loss of *FLC*:*GUS* expression. Although the male gametes do not express *FLC*:*GUS*, expression is detected in the single-cell zygote. *FLC*:*GUS* activity occurs around the time that has been reported for the DNA replication-independent removal of the male gamete-specific histone H3 variant (23). *FLC*:*GUS* expression does not occur in the syncytial endosperm, indicating that the second male gamete that fertilizes the central cell, although identical to the sperm cell fertilizing the egg cell, does not express *FLC*:*GUS*, perhaps because of the absence of required transcriptional activators.

In contrast to *FLC*:*GUS* expression during male gametophyte development and expression of the paternally derived gene in the zygote, we did not observe *FLC*:*GUS* expression during female gametogenesis, nor did we observe expression of the female-derived gene in the zygote. The first detectable expression occurred several cell divisions later in the early multicellular embryo. The differential timing of expression shows that there must be differences in the *FLC* chromatin of the maternal and paternal gametes. The delay in expression of the female-derived gene may indicate that the chromatin is still in the vernalization-repressed state at the zygote stage and that the modified histones are passively diluted out during the zygotic and early-embryo mitotic divisions. Passive loss of histone modifications could occur because of loss of activity of genes such as *VRN2*, which is required for the repressive histone modifications at *FLC* in response to vernalization (6, 8).

We have uncovered a regulatory activity whereby reduced endogenous *FLC* expression in early-globular embryos directly or indirectly results in an increased expression of the paternally derived *FLC*:*GUS* transgene. Consistent with the vernalized

maternally inherited *FLC* gene not being active until the early-globular stage, there was higher expression of the male *FLC*:*GUS* gene in embryos on vernalized plants compared with nonvernalized plants.

This difference in *FLC* expression in embryos of vernalized and nonvernalized plants has no effect on flowering time (data not shown), indicating that early embryo FLC is not essential for flowering time control. *FLC* is expressed throughout embryo development; however, it is not essential for embryo development as *flc*-null mutant embryos develop normally. Data from the inducible construct suggest that *FLC* expression during late embryogenesis is required for delayed flowering of the adult plant. In the late-stage embryo *FLC*:*GUS* expression is localized to the embryonic provascular cells. Constructs that direct *FLC* expression in the embryonic provascular cells are able to confer a delay in flowering, whereas a construct with expression limited to the shoot meristem zone causes a reduced delay in flowering. *FLC* expression may be required to repress *FT*, thereby preventing movement of FT protein to the apex and avoiding precocious flowering.

Materials and Methods

Plant Lines and Constructs. Plants were grown as described (24), except that a 56-day cold treatment was used. *FLC-C24*:*GUS* construct in C24 ecotype contains the C24 genomic *FLC* sequence with GUS replacing the stop codon (SI Fig. 6). Preliminary experiments were conducted with three independent lines, with all three lines giving similar results. The data presented derive from one line. This line was crossed into the *flc-20* mutant (15) and homozygous F_2 plants selected by flowering time and PCR testing. One line of L*er FRI* + *FLC-Col*:*GUS* was used for the data presented. *FLC-Col*:*GUS* contains *FLC*-Col fused to the GUS gene at an NheI site in exon 6 (8). The *FLC*-Col has been characterized genetically as having higher activity than the C24 allele (25). L*er FRI* was generated by introducing a genomic clone of *FRI*-H51 into L*er*. L*er FRI*-Sf2 was obtained from R. Amasino (University of Wisconsin, Madison, WI). For dexamethasone induction of *FLC* and misexpression of *FLC*, the amplified FLC-1/FLC-2 coding region (for primers see SI Table 2) was cloned into pVTOP (13) and transformed into L*er* to generate *pOp-FLC*/*GUS*. Homozygous T3 lines were supertransformed with pBIN 35S:LhGR-N (13). Lines homozygous for both constructs (*LhGR*/*pOp-FLC*/*GUS*) were selected. Dexamethasone (10 μM ; Sigma–Aldrich) in 0.1% ethanol was used in MS agar for induction of imbibed seed and seedlings. Flowering plants in soil were dipped in 10 μM dexamethasone, 0.1% ethanol, and 0.03% silwet-L77 (Lehle Seeds). Control plants were treated with 0.1% ethanol. Coinduction of both FLC and GUS was confirmed by immunodetection of FLC and histochemical analysis for GUS (26). Misexpression activator lines in L*er* were obtained from J. Bowman and P. Brewer (Monash University, Melbourne, Australia). pANT and pCLV1 are described by Schoof *et al.* (27). pCUC2 contains 3.48 kb, pSUC2 contains 3.9 kb, and pSTM contains 7.2 kb upstream of the ATG. *pOp-FLC*/*GUS* was used as the reporter line.

Histochemical GUS Imaging. GUS staining was carried out as described by Sheldon *et al.* (28). After staining, dissected embryos and seedlings were cleared in ethanol and ovules were cleared in 8:1:3 chloral hydrate:glycerol: water or 20% lactic acid/20% glycerol. Plants were photographed by using a Leitz M8 dissecting microscope with a Colorview Soft Imaging System camera (Leitz). Embryos and ovules were imaged by using DIC optics on a Leica DMR upright microscope with DC500 camera (Leica). After GUS staining, buds were fixed in 3% glutaraldehyde, dehydrated through an ethanol series, and embedded in LR White resin. One-micrometer transverse sections were cut and imaged under dark-field conditions.

mRNA Expression Analysis. RNA extraction and gel blot analysis were as described (24). Real-time RT-PCR was carried out as described (7), using primers shown in SI Table 2. Expression data were normalized against expression of the *FDH*.

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