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Major flowering time gene, FLOWERING LOCUS C, regulates seed germination in Arabidopsis thaliana

George C. K. Chiang, Deepak Barua, Elena M. Kramer, Richard M. Amasino, and Kathleen Donohue

*Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138; †Department of Biochemistry, University of Wisconsin, Madison, WI 53706; and ‡Department of Biology, Duke University, Durham, NC 27708

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FLOWERING LOCUS C (FLC) is a major regulator of flowering responses to seasonal environmental factors. Here, we document that FLC also regulates another major life-history transition-seed germination, and that natural variation at the FLC locus and in FLC expression is associated with natural variation in temperature-dependent germination. FLC-mediated germination acts through additional genes in the flowering pathway (FT, SOC1, and AP1) before involving the abscisic acid catabolic pathway (via CYP707A2) and gibberellins biosynthetic pathway (via GA20ox1) in seeds. Also, FLC regulation of germination is largely maternally controlled, with FLC expression in seeds being high (87–100%) in FRI mutants and low (5–10%) in FRI-1 plants, indicating that gene expression before the physiological independence of seeds can influence gene expression well after any physical connection between maternal plants and seeds exists. The major role of FLC in temperature-dependent germination documented here reveals a much broader adaptive significance of natural variation in FLC. Therefore, pleiotropy between these major life stages likely influences patterns of natural selection on this important gene, making FLC a promising case for examining how pleiotropy influences adaptive evolution.

life history | pleiotropy | vernalization | natural variation | FRIGIDA

One goal toward understanding the genetic basis of adaptation is to accurately interpret geographic patterns of genetic variation in terms of local adaptation. A major challenge in this agenda is to determine the relationship between variable genes and the phenotypes that are exposed to natural selection. A single gene may influence >1 trait, and this pleiotropy may be a considerable constraint on adaptive evolution. The extent to which pleiotropy actually constrains local adaptation depends on how divergent or convergent the complete pathways that regulate the expression of different traits are. Therefore, it is necessary to evaluate the extent of pleiotropy expressed by naturally variable genes, and to characterize the degree of convergence in pathways that regulate the expression of different traits under selection. In this study, we found that the gene FLOWERING LOCUS C (FLC) (1, 2), which has previously been shown to be associated primarily with the developmental transition to reproduction, is also strongly associated with the earlier developmental transition of germination.

Among the best characterized genetic pathways regulating a complex life-history trait in plants is the flowering pathway of Arabidopsis thaliana (3–6). A central gene in this pathway is FLC (1, 2), which represses several loci that promote flowering. FLC repression of flowering is relieved by an epigenetic switch that represses the expression of FLC itself via chromatin remodeling (5). FLC repression can occur after plants experience an extended period of cold (vernalization) (7, 8), or, alternatively, by the autonomous pathway, which senses plant age and ambient temperature (5, 9). It has been established that FLC expression is promoted by the gene FRIGIDA (FRI), although the exact biochemical basis of the function of FRI remains unknown (8). As a MADS-box transcription factor (so named for MCM1, AGAMOUS, DEFICIENS, and serum-response factor), FLC directly binds to and inhibits expression of the downstream genes of SOC1 and FT (10, 11). The repression of these genes, in turn, delays expression of the floral meristem identity genes LEAFY (LFY) and APETALA1 (API) to prolong the vegetative phase (12–15). Natural allelic variants of FLC are associated with variation in flowering time, either singly (16–18) or in combination with natural loss of function alleles of FRI (1, 2, 19–21).

Variation in these 2 genes has been shown to account for major differences between the winter annual and the rapid-cycling life histories of Arabidopsis. Because FLC has such a large effect on flowering, the maintenance of natural variation in FLC has been thought to be primarily due to geographically variable natural selection on flowering time (19, 22–26). Therefore, FLC is unique for ecological studies in that it is one of very few genes for which we have molecular pathway data, natural variation data, and abundant hypotheses concerning how the two are related.

Although much is known about the seasonal and genetic regulation of flowering time, far less is known about the ecological and genetic basis of germination, an earlier life-history developmental transition (27, 28). However, germination timing is under intense natural selection; it has been shown to account for >70% of the variation in fitness among genotypes in an experimental population of recombinant inbred lines (29), and it is the life-stage transition that has the largest effect on projected population growth rates in natural populations of Arabidopsis (30). Also, new evidence suggests that the seasonal timing of germination might be a stronger factor in determining the flowering time of Arabidopsis in the field than variation in the genetic basis of flowering time itself (31). As sessile organisms, the primary way plants determine the seasonal environment of their establishment and subsequent growth is through germination cues. Therefore, genes associated with natural variation in germination are likely to be under strong natural selection.

Germination and flowering are both fundamental developmental transitions that require precise environmental sensing and responses to multiple seasonal cues to accurately match developmental timing to appropriate seasonal conditions. The combination of these 2 phenological traits determines the overall life cycle and generation time of many plants, including A. thaliana (32–35). Because both transitions respond to similar seasonal cues, it is logical to hypothesize that genetic pathways...
of these 2 life-history transitions share common elements. However, each developmental transition necessarily occurs during different seasons, so some divergence in their regulation is also required. Here, we report that a major regulator of flowering, FLC, controls the germination behavior of seeds in a temperature-dependent fashion, and that natural variation in FLC alleles and FLC expression is associated with natural variation in temperature-dependent germination. Also, we showed that FLC regulation of germination is largely maternally controlled and occurs at later stages of seed maturation, and that the signal is transmitted through the downstream pathway involving FT, SOC1, and AP1 to connect with the abscisic acid (ABA) and gibberellins (GA) germination pathways in seeds.

Results

FLC Controls Temperature-Dependent Seed Germination. Natural genetic variants and genetic constructs of A. thaliana with high levels of FLC expression exhibited significantly higher germination percentages at cool (10 °C), but not warm (22 °C), temperatures (Fig. 1). We used near isogenic lines (NILs) containing different natural alleles of FLC in the Landsberg erecta (Ler) background that naturally has a low-expressing FLC allele (16) and a nonfunctional FRI allele. When seeds were matured under cool temperatures (15 °C), resembling ecologically realistic seed maturation conditions during spring or autumn, the Ler background line exhibited high dormancy. This dormancy was observed when seeds were imbibed at either cool or warm temperatures (in a 12-h light/12-h dark cycle for 15 days). In contrast, a line containing an introgressed high-expressing FLC allele (17) from the Cape Verde Island ecotype (NIL-FLC_Cvi) had a significantly higher proportion of germination when seeds were imbibed at cool temperatures, but only slightly higher germination at warmer temperature (Fig. 1A). Although the difference of germination at 22 °C between Ler and NIL-FLC_Cvi was statistically significant, the increase was extremely small so it is not likely to be biologically relevant. A similar effect on germination was seen in a second high-expressing FLC NIL that contained the FLC allele from the Columbia (Col) ecotype and a functional FRI allele from the San Felio ecotype (NIL-FRIs+FLC_Col). Therefore, the observed germination phenotype of increased germination at cool, but not at warm temperature, may be caused by highly expressed natural FLC alleles. Also, FLC-mediated germination apparently does not require functional FRI, at least with the FLC allele from the Cape Verde Island ecotype (Cvi), as indicated by the similar germination phenotype of the 2 NILs (one that has a functional FRI and one that does not), even though FLC expression in the seedling stage is >5 times greater with the functional FRI allele (Fig. S1).

To examine whether this temperature-dependent, FLC-mediated germination behavior is also seen in a natural seasonal environment, freshly matured seeds of NIL-FLC_Cvi and its background Ler-WT were dispersed into a field site in Bedford, Massachusetts, during mid October when the daily temperature was fluctuating ~10 °C (average daily high and low: 14 and 4 °C). The NIL-FLC_Cvi germinated to significantly higher proportions during October and November compared with its background Ler-WT (Fig. 1A). Therefore, FLC has the potential to be a major influence on germination of A. thaliana under complex natural field conditions.

We verified that FLC was causally implicated in germination, as opposed to another gene within the introgressed regions of the NILs, by examining the germination of a transgenic line overexpressing FLC under the control of the constitutive 35S promoter (35S::FLC) (36). The FLC-overexpressor exhibited much higher germination compared with the Col background genotype (Fig. 1B) at cool (10 °C), but not at warm (22 °C) temperatures, as in the NILs. This mediation of germination by high levels of FLC expression could be initiated with any combination of sequential temperature fluctuations resembling seasonal changes that involve a cold period (Fig. S2), and the germination proportion increased with an increased duration of cold, and could be saturated with 3 days of 10 °C (Fig. S3). Also, FLC-promoted germination required light (Fig. S2).

Natural Variation in FLC Expression Is Associated with Natural Variation in Germination. Further implication of FLC in germination at cool temperature was apparent from a survey of 52 ecotypes collected from the native range of A. thaliana in Eurasia and Africa. Because photoperiod and temperature both vary seasonally, we matured seeds under long day (14-h light/10-h dark) and short day (10-h light/14-h dark) conditions, and measured germination of seeds imbibed at 10 and 22 °C (in a 12-h light/12-h dark cycle). Using published data on levels of FLC expression in vegetative rosettes (20), and controlling for population structure (37), we found that higher FLC expression was significantly associated with increased germination when seeds were imbibed at 10 °C, but not at 22 °C (Fig. 2), consistent with the results from the NILs and overexpressor lines. We found no effect of photoperiod during seed maturation on the relationship between FLC and germination. These results suggest that variation in FLC expression is associated with natural variation in germination at low temperature. This knowledge greatly revises our expectations of natural selection on FLC and our interpretation...
This maternal control of FLC germination phenotypes, because heterozygotes containing a high-

**Fig. 3.** Maternal control of FLC-mediated germination. (A) Proportion of viable seed germination (± 1 SE) at 10 °C for fresh seeds from reciprocal crosses (Ler−/ Ler− × NIL-FLC_Cvi, · · ·; FLC−/ NIL-FLC_Cvi × Ler−/ Ler−; · · · − self-cross). Asterisks indicate significant differences between 2 adjacent bars. *P < 0.05; **P < 0.01. (B) Relative FLC expression (±1 SE, normalized by ACT8) at different stages during development of NIL-FLC_Cvi (closed circles) and Ler-WT (open circles). BB, rosette before bolting; AB, rosette after bolting; Inflo, inflorescence; 5–20 d s, 5–20-day-old siliques after flower opening; Seed, fresh dry seed; 10C-1d, 1 day of imbibition at 10 °C. The peak of FLC expression in NIL-FLC_Cvi is significantly different (P < 0.05) from all other time points except those in seeds, 10 °C-1d, 10 °C-3d, and 22 °C-3d, based on Tukey’s post hoc tests.

of the geographic distribution of FLC variation, which have primarily focused on the influence of FLC on flowering time.

**Maternal Determination of the FLC Germination Phenotype.** To determine whether FLC-mediated germination is controlled maternally or solely by the embryonic genotype, reciprocal crosses were performed between Ler-WT and NIL-FLC_Cvi, and between Col-WT and the 35S::FLC overexpressor (Fig. 3A; Figs. S4 and S5). If FLC regulation of germination is exclusively under embryonic control, germination should not differ between the reciprocal heterozygous offspring, because they both would have one copy of the stronger FLC-allele. In contrast, if FLC-regulated germination is under maternal control, heterozygotes derived from the strong FLC maternal plants would have higher germination at cool temperatures than heterozygotes derived from weak FLC maternal plants. The results indicate that FLC-mediated germination is largely controlled by the maternal genotype, because heterozygotes containing a high-FLC expressing maternal allele had higher germination at 10 °C than heterozygotes containing a high-FLC-expressing paternal allele. This maternal control of FLC-mediated germination could be through either FLC expression in the maternal tissue (in siliques or funiculus) or preferential expression of maternal FLC alleles in the seeds.

The asymmetrical contribution of the maternal FLC allele, observed from the reciprocal crosses, suggested that the effect of the high-expressing FLC allele on germination may be most prevalent during seed maturation when the physical connection between the maternal plant and the developing seed is strong. To further narrow down the possible developmental stages when this FLC effect might be imposed, FLC expression of the NIL-FLC_Cvi was followed throughout the life cycle, especially during silique development (seed maturation) and seed germination. FLC expression increased substantially midway through silique development, and peaked at the later stage of seed development when the siliques were starting to turn yellow before seed dispersal (Fig. 3B). This stage is also when prominent dormancy genes, such as DOG1, are most highly expressed and when primary dormancy is induced (38, 39). FLC mRNA levels decreased subsequently, but remained relatively high in dry seeds and in seeds imbibed at both 10 and 22 °C, with no obvious pattern of change that correlated with germination differences. FLC expression in the Ler-WT remained relatively low during late silique development, dry seed, and seed imbibition stages, and the pattern of an FLC expression peak late in silique development was not seen, in contrast to the pattern found in NIL-FLC_Cvi (Fig. 3B).

**Parallel Pathways of FLC-Mediated Flowering and Germination.** In the flowering pathway, FLC represses the genes FT and SOC1, causing low expression of AP1 and consequently inhibiting flowering. If FLC regulation of germination shares this pathway, mutants in these genes should recapitulate the germination phenotype seen in high FLC-expressing genotypes; namely, higher germination at low, but not warm temperatures. Supporting this scenario, the apo1-1 mutant, which is in the Ler background, showed significantly higher germination at low, but not warm temperatures compared with the Ler-WT (Fig. 4A), similar to results from the NIL-FLC_Cvi NIL-FRI_Sf and FLC_Col, and the 35S overexpressor lines. Moreover, ft-1 and soc1 mutants also had higher germination percentages at cool temperatures than Ler-WT, but not as high as the apo1-1 mutant, indicating that they might act redundantly downstream of FLC. Further evidence of a shared pathway between flowering and germination is demonstrated by the significantly greater down-regulation of AP1, FT, and SOC1 in NIL-FLC_Cvi late during silique development.
compared with the expression of these genes in the weak FLC background Ler (Fig. 4 B–D).

Connecting Flowering and Germination Pathways. A seed needs to break dormancy before it can successfully germinate (40). The 2 primary plant hormones regulating dormancy and germination are ABA, which induces and maintains dormancy, and GA, which stimulates germination (40–42). Endogenous levels of these 2 hormones largely determine whether a seed will germinate. Therefore, we hypothesized that FLC regulates germination via the biosynthesis or catabolism of one or both of these hormones.

To test whether the difference in germination of different FLC genotypes was due to differences in the synthesis of ABA or GA by imbibed seeds during the initial stages of germination, uniconazol and fluridone, which inhibit the synthesis of GA and ABA, respectively, were applied to imbibing seeds. Uniconazol completely prevented germination of both NIL-FLC_Cvi and 35S::FLC, as well as their WT background at 10 °C, indicating that FLC-mediated germination in cool temperatures cannot overcome the lack of further GA synthesis during germination (Fig. 5A). Fluridone did not alter the germination behavior of any genotypes under the same conditions, indicating that ABA synthesis during imbibition is not the major factor that distinguishes FLC-mediated germination phenotypes.

To test whether differences in FLC expression predict differences in downstream hormonal pathways associated with germination, we measured the expression of genes involved with ABA and GA synthesis and catabolism in developing siliques and imbibing seeds (Table S2). Several of the genes we investigated did not have significant expression differences between NIL-FLC_Cvi and Ler-WT. In contrast, expression of CYP707A2, one of the major ABA degradation genes, was significantly higher in genotypes with a strong FLC allele after 1 day of imbibition at 10 °C (Fig. 5B). Also, the major gene in the early stages of the GA synthesis pathway GA20ox1, increased dramatically in NIL-FLC_Cvi after 3 days of imbibition at 10 °C (Fig. 5C).

A similar trend in the differential expression of CYP707A2 and GA20ox1 was also observed in seeds from a 35S::FLC line compared with its low FLC-expressing Col background (Fig. S6). GA3ox1, a gene that regulates the conversion of inactive to bioactive GA, also was expressed to higher levels in high FLC-expressing lines after 3 days of imbibition at 10 °C, but this increase was not statistically significant. The results suggest that a strong FLC allele promotes the early degradation of endogenous ABA followed by additional GA synthesis in imbibed seeds that experience cold (Fig. 5D). This process could occur either through the parallel sequential control of the 2 hormonal pathways by downstream components of the FLC pathway, or by FLC-pathway-mediated ABA degradation and subsequent dormancy breakage, which then enables GA-promoted radial protrusion and germination.

Discussion

The major flowering time gene FLC promotes temperature-dependent seed germination. Therefore, significant pleiotropy exists between the 2 fundamental developmental transitions of germination and flowering, both of which require precise sensing and responses to seasonal cues. Shared pathways of flowering and germination occur, with FLC-mediation of germination acting through FT, SOC1, and AP1, similar to what occurs in FLC-mediated flowering. FLC regulation of germination subsequently acts through the pathway of ABA degradation and GA synthesis in imbibed seeds. Significant components of the same genetic pathway are shared between flowering and germination regulation, but downstream divergence may occur. Identifying precisely when the pathways diverge would contribute significantly to our understanding both of the genetic basis of pleiotropy of these life-history traits and of the importance of pleiotropy as a constraint on adaptive phenotypic expression of multiple traits.

It is interesting to note that functional FRIGIDA (FRI) does not appear to be required for FLC-mediated germination for some FLC alleles, because the 2 NILs that contained functional FLC both had similarly increased germination even though one of them had nonfunctional FRI. Therefore, some difference in the pathways of flowering and germination may also exist upstream of FLC. It is possible that the threshold of FLC expression for germination is lower in the Ler background. Alternatively, the genes regulating FLC in rosettes during the transition to reproduction might differ or might have different effects on FLC expression from those acting during seed maturation. Vegetative FLC expression level was nevertheless a good predictor of germination in a sample of natural ecotypes.

FLC in vegetative rosettes is epigenetically silenced through chromatin remodeling during an extended period of cold, and FLC activity is reset during the early reproductive phase (9, 43–46). In contrast, to elicit germination, both high FLC ex-

![Fig. 5.](image-url)
expression and cool temperatures are required. The temporal separation of high FLC expression (during seed maturation) and cool temperature (during imbibition) can account for the requirements for both high FLC and cool temperature; FLC itself need not be expressed during low-temperature imbibition. It appears that increased FLC expression may impede both flowering and dormancy. We propose that high FLC expression during seed maturation renders seeds able to overcome primary dormancy during imbibition, most likely via lowering endogenous ABA levels, followed by GA-promoted germination (Fig. 5D).

FLC regulation of germination is largely maternally controlled. This maternal control could be imposed during the late stages of seed development, when FLC expression peaked and corresponding levels of FT, SOC1, and API decreased. Interestingly, FLC expression during seed development seems to alter the expression of hormonal genes in imbibed seeds, long after any physical connection between the maternal plant and seeds. One possibility for the maternal control of FLC-mediated germination is that mRNA and proteins involved in dormancy and germination are synthesized directly in seeds via the expression of maternal FLC alleles. Alternatively, high maternal FLC expression during silique development may prime seeds to be responsive to cold in terms of promoting germination through chromatin remodeling or other means of epigenetic manipulation. Also, mRNA transcripts, hormones, or proteins could have been provisioned to seeds during late stages of seed maturation, and these compounds may either degrade (reducing dormancy maintenance) or become active (promoting germination) during imbibition (47, 48). Stored RNAs have been shown to degrade during seed germination as a result of transcription during imbibition (49), but seeds are metabolically active even before imbibition, because transcription (50–52), protein synthesis (53), and metabolism (54, 55) occur even in dry seeds. The role of stored compounds in regulating dormancy and germination is perhaps one of the least understood phenomena in seed biology; FLC regulation of germination may offer a promising system for investigating these processes in a comparatively well-characterized genetic pathway.

The major role of FLC in temperature-dependent germination documented here indicates that a reevaluation of the adaptive significance of natural variation in FLC is required. Evolutionary ecological studies of FLC have focused almost exclusively on the role of FLC and its epistatic interactions with FRi to produce variation in flowering time, and they have interpreted natural variation in FLC in terms of variable natural selection on flowering time. Although evidence for natural selection on flowering phenology has been shown to be among the strongest documented for a single life history trait (29, 30, 56). It is likely that natural variation in FLC and other genes in its pathway may reflect a history of natural selection on germination as much as, if not more than, natural selection on flowering. This hypothesis needs testing in the field.

FLC regulation of flowering and germination provides an excellent system for investigating how pleiotropy contributes to or constrains adaptive evolution. Although we could predict that one gene that regulates 2 life history traits with different phenotypic optima may necessarily impose adaptive challenges, pleiotropy may actually contribute to the coordinated expression of different traits. For example, many of the ecotypes with high FLC expression are late-flowering winter annuals from colder climates. In these winter annuals, seeds likely germinate in cool temperatures of autumn and postpone flowering until after vernalization. As such, high FLC expression would enable both germination in autumn and flowering in spring, and thus, may actually be an example of adaptive pleiotropy that contributes to local adaptation to colder climates. Further study of the convergence and divergence of FLC-mediated flowering and germination pathways, combined with explicit tests of the adaptive value of pleiotropic control of these fundamental developmental transitions, would provide insight into the genetic basis of adaptation, given the constraints of shared genetic pathways across multiple traits.

Materials and Methods

NIL-FLCer (named LCNS-1) and its Ler background were previously described (57–59). The NIL-FLCer and FLCer in the Ler background and 35S::FLC in the Col background, as well as their corresponding WTs, were previously described (8). Table 51 lists the ecotypes used in this study and their original sites of collection. All of the ecotypes and the flowering time mutants fr-1 (CS56), soc1 (CS27278), and api-1 (CS28) were obtained from the Arabidopsis Biological Resource Center seed stock center. Experimental seeds were matured in 10-h light/14-h dark at 15 °C. Germination assays were conducted with fresh seeds placed on 0.5% agar. Three independent biological replicates were used for qRT-PCR analysis (Table S2). RNA extractions were performed according to ref. 60, with the following modification: only 1 round of phenol extraction was performed. Transcript levels were calculated and presented with normalization to ACT18 using a cDNA dilution series for each primer set in each experiment. Similar expression patterns were reconfirmed with the TUB2. For more details, see SI Materials and Methods.

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