USING NEAR-ISOGENIC LINES TO DISSECT THE FLOWERING BEHAVIOR OF A NATURAL ACCESSION OF *Arabidopsis thaliana*

by

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Biochemistry)

at the

UNIVERSITY OF WISCONSIN–MADISON

2009

Special thanks to my husband, Nicolas Addington, and to UW–Madison undergraduates Jennifer Kraninger, Libby Karn, Meghan Truckey, Ellen Tangel, Curren Sharma, Leah Burmeister, and Charles Calderón.

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ABSTRACT

In well-characterized laboratory strains of *Arabidopsis*, a late-flowering phenotype depends upon the possession of strong alleles of both *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*). In these strains, flowering is accelerated by the experience of prolonged winter cold, which, during a process called vernalization, acts to stably reduce *FLC* expression. However, many naturally-occurring variants of *Arabidopsis*, called accessions, exhibit alternate flowering behaviors, the molecular explanations of which remain unknown. In this study, we begin work with Tul-0, an accession that exhibits two unusual flowering phenotypes: first, it is late-flowering despite possessing a weak *FLC* allele, and, second, it remains late-flowering following vernalization. Using near-isogenic lines (NILs), created both by the introgression of Tul-0 loci into laboratory accessions and by the introgression of loci from laboratory accessions into Tul-0, we begin to dissect these phenotypes and to define roles for multiple Tul-0 loci. We show that late flowering is conferred by both *FLC*-independent and *FLC*-dependent mechanisms and, at the same time, document the existence of new *FLC*-enhancer loci, that, in addition to *FRI*, contribute to natural variation. Finally, we show that Tul-0's reduced vernalization sensitivity is genetically separable from late flowering per se and that it is accompanied by changes in vernalization-associated *FLC* silencing.

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CHAPTER 1

THE CONTROL OF FLOWERING TIME IN *Arabidopsis thaliana*: AN INTRODUCTORY LITERATURE REVIEW

“Betrachten wir eine Pflanze in sofern sie ihre Lebenskraft äussert, so sehen wir dieses auf eine doppelte Art geschehen, zuerst, durch das Wachstum indem sie Stengel und Blätter hervorbringt, und sodann durch die Fortpflanzung, welche in dem Blüten- und Fruchtbau vollendet wird.”

“Umständen eine Pflanze nötigen, dass sie immerfort sprose, man kann dagegen den Blütenstand beschleunigen.”

-Johann Wolfgang von Goethe, Versuch die Metamorphose der Pflanzen zu erklären, 1790

INTRODUCTION

In many species, it is important to coordinate reproduction with the appearance of favorable environmental conditions. Ill-timed reproduction may, at best, constitute an inefficient use of energy, or, at worst, lead to offspring failure. The coordination of reproduction with external conditions may be particularly important for plants, which, being sessile, cannot, at will, exchange their given microenvironment for another. In plants, the transition from vegetative to reproductive growth is marked by the production of the first flower, which contains the reproductive organs. In order to coordinate reproduction with optimal conditions, many plant species predicate flower initiation upon the receipt of seasonal cues, the most prominent of which are day length and temperature.

The question of how, on a molecular level, the receipt of these seasonal cues is linked to flower initiation has been undertaken with particular success in the model plant *Arabidopsis thaliana*. *Arabidopsis* is a favored laboratory organism because it is small—both physically and genomically—has a rapid generation time, and, compared to other plants species, is easy to manipulate genetically. Experiments in *Arabidopsis* have shown that light and temperature cues are processed via distinct pathways but that these pathways converge, downstream, on a common set of floral integrators. These floral integrators, in turn, signal a cessation in the production of vegetative leaves and a transition to the production of flowers. Experiments in *Arabidopsis* have also shown that the timing of flowering is influenced not only by external cues but also by endogenous factors, which also act by modulating levels of the floral integrators.

Many of these experiments have depended upon mutagenized populations, which have been screened to identify mutants exhibiting alternate flowering phenotypes. Another important

resource has been the existence of numerous genetically distinct natural populations of *Arabidopsis*, some of which exhibit different flowering behaviors. Genomic regions—and, in some cases, the causative genes—associated with flowering behaviors in natural populations can be identified using a variety of mapping techniques. Experiments using natural rather than mutagenized populations also have the potential advantage of pertaining directly to evolutionary questions.

DAY LENGTH PERCEPTION AND THE FLORAL INTEGRATORS

Arabidopsis is a long day plant, meaning that, in *Arabidopsis*, flowering is accelerated by the long days of spring and summer and inhibited by the short days of fall and winter. The perception of day length occurs in the leaves, where it leads to the accumulation of the floral promoter CONSTANS (CO), a zinc finger protein that may act as a transcription factor (Puterill et al. 1995). Levels of *CO* mRNA cycle throughout the day, under the control of the circadian clock; meanwhile, CO protein is stabilized in the light and degraded by the proteasome in the dark, a distinction which is perceived by the photoreceptors PHYTOCHROME A and B and CRYPTOCHROME 1 and 2 (Suárez-López et al. 2001; Valverde et al. 2004). The effect of these two layers of regulation is that high levels of CO protein are present only under long day conditions, when high levels of *CO* mRNA coincide with conditions conducive to protein stability.

High levels of CO elevate the expression of the floral integrator *FT*, which encodes a RAF kinase inhibitor-like protein (An et al. 2004; Samach et al. 2000; Kardailsky et al. 1999; Kobayashi et al. 1999). From the leaves, FT travels to the shoot apical meristem (SAM), where it complexes with the bZIP transcription factor FD (Corbesier et al. 2007; Jaeger and Wigge

2007; Abe et al. 2005; Wigge et al. 2005). Together, FT and FD activate the expression of the floral integrators *APETALA 1* (*AP1*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Abe et al. 2005; Wigge et al. 2005; Michaels et al. 2005; Yoo et al. 2005).

SOC1 activates the expression of *AGAMOUS-LIKE 24* (*AGL24*) and, together with AGL24, activates expression of the floral integrator *LEAFY* (*LFY*) (Liu et al. 2008; Lee et al. 2008). LFY, in turn, activates genes required for actual flower formation, including *AP1*, *APETALA 3* (AP3), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) (Liljegren et al. 1999; Wagner et al. 1999; Sablowski 2007). Interestingly, SOC1 and AGL24 upregulate one another's expression, as do LFY and AP1, and it has been suggested that these cycles of mutual upregulation, which amplify the initial signal, may contribute to the irreversibility of the transition from vegetative to reproductive growth (Liu et al. 2008; Liljegren et al. 1999; Wagner et al. 1999).

VERNALIZATION AND *FLC*

In *Arabidopsis*, flowering is accelerated not only by the long day conditions, which serve as a signal that spring has arrived, but also by the experience of prolonged winter cold, which serves as a signal that winter has occurred. The acceleration of the capacity to flower by the experience of prolonged cold is called "vernalization," a term derived from "vernum," the Latin word for spring (Chouard 1960). A vernalization response is distinct from short-term cold responses such as cold shock or seed stratification because vernalization requires an extended period of cold, on the order of several weeks. This requirement for long term cold is important because it enables the plant to distinguish between a brief cold spell in fall, which presages only winter, and winter itself, which truly presages spring.

In *Arabidopsis*, vernalization accelerates flowering by reducing expression of the floral repressor *FLOWERING LOCUS C* (*FLC*), which encodes a MADS box transcription factor (Sheldon et al. 1999; Michaels and Amasino 1999). FLC forms a heterodimer with the MADS domain protein SHORT VEGETATIVE PHASE (SVP), whose levels are unchanged by vernalization, and formation of this heterodimer is required in order for FLC to confer a late-flowering phenotype prior to vernalization (Li et al. 2008; Lee et al. 2007; Fujiwara et al. 2008). In complex with SVP, FLC delays flowering by directly repressing the expression of the floral integrators *FT*, *FD*, and *SOC1* (Hepworth et al. 2002; Michaels et al. 2005; Searle et al. 2006; Helliwell et al. 2006).

The reduction of *FLC* expression during vernalization is accompanied by a reduction in the levels of active chromatin marks and by an increase in the levels of the repressive histone H3 modifications H3K9 and H3K27 dimethylation at *FLC* chromatin (Bastow et al. 2004; Sung and Amasino 2004). Following vernalization, these repressive histone marks persist. The persistence of these marks is correlated with the stability of *FLC* repression: in some mutants, these repressive marks do not persist, and, in such mutants, *FLC* repression is not stable and flowering time is not accelerated (Bastow et al. 2004; Sung and Amasino 2004; Mylne et al. 2006; Sung et al. 2006a and b; Greb et al. 2006).

Several proteins are required for the vernalization-mediated silencing of *FLC* via the deposition or maintenance of repressive marks at *FLC* chromatin. One of these proteins, VERNALIZATION INSENSITIVE 3 (VIN3), contains a plant homeodomain (PHD) and fibronectin 3 (FNIII) domain (Sung and Amasino 2004). *VIN3* exhibits a vernalization-specific expression pattern: *VIN3* is elevated only following prolonged cold and drops again to low levels following the return to warm conditions (Sung and Amasino 2004). This vernalization-specific

expression distinguishes *VIN3* from the genes encoding other components of the vernalization-required machinery, which are expressed constitutively, and, for this reason, VIN3 is regarded as the most upstream component of the vernalization pathway. However, constitutive *VIN3* expression is not sufficient to produce a vernalization response, indicating that additional cold-induced factors, which may act in parallel, are also required (Sung and Amasino 2004). In addition, what might lie upstream of *VIN3* itself, i.e., what regulates *VIN3* expression at the molecular level and activates its expression only following prolonged cold, also remains to be determined.

The VIN3 relative VIN3-LIKE 1/VERNALIZATION 5 (VIL1/VRN5) is also required for vernalization-mediated *FLC*-repression, but, unlike VIN3, VIL1/VRN5 does not show a vernalization-specific expression pattern (Sung et al. 2006b; Greb et al. 2007). Another required protein is VERNALIZATION 2 (VRN2), a homolog of Suppressor of Zeste 12 (Su(Z)12), which is a component of the transcription regulatory complex Polycomb Repressive Complex 2 (PRC2) in *Drosophila* (Gendall et al. 2001). Vernalization-mediated *FLC* repression also requires VERNALIZATION 1 VRN1, a non-sequence specific DNA binding protein, and LIKE HETEROCHROMATIN PROTEIN 1/TERMINAL FLOWER 2 (LHP1/TFL2), a homolog of the chromatin binding protein HP1 that has been characterized in other systems (Levy et al. 2002; Sung et al. 2006a; Mylne et al. 2006).

The fact that VRN2 is homologous to the PRC2 component Su(Z)12 provided the first indication that a PRC2-like complex might repress *FLC* expression following vernalization. In support of this hypothesis, in *Arabidopsis*, homologs of additional components of the Drosophila PRC2 complex have been shown to associate with VRN2 and to form a complex whose levels increase following vernalization (Wood et al. 2006; De Lucia et al. 2008). Components of this

PRC2-like complex include FERTILIZATION INDEPENDENT ENDOSPERM (FIE), which is homologous to the PRC2 component Extra Sex Combs (ESC), MULTICOPY SUPPRESSOR OF IRA 1 (MSI1), which is homologous to the PRC2 component P55, and SWINGER (SWN) and CURLY LEAF (CLF), which are homologous to the PRC2 component Enhancer of Zeste (E(Z)) (Ohad et al. 1999; Köhler et al. 2003; Chanvivattana et al. 2004). The PHD proteins VIN3, VIL1/VRN5, and an additional VIN3 relative, VEL1/VIL2, have also been shown to associate with this complex (Wood et al. 2006; De Lucia et al. 2008). In addition, *FLC* itself contains a region in the middle of the first intron, called the Vernalization Response Element (VRE), which is required for vernalization-mediated silencing. It has been suggested that the VRE may function in a manner equivalent to the Polycomb Response Element (PRE) in *Drosophila*, which is required in cis for targeting of a given gene by PRC2 (Sung et al. 2006a). These facts together support a model in which a PRC2-like complex, in association with the PHD proteins VIN3 and VIL1/VRN5, is responsible the vernalization-induced silencing of *FLC*.

Vernalization also alters the expression of the closest relatives of *FLC*, which include *MADS AFFECTING FLOWERING 1/FLOWERNG LOCUS M* (*MAF1*/*FLM*) and *MADS AFFECTING FLOWERING 2-5* (*MAF2-5*), all of which, like *FLC*, appear to encode floral repressors. Interestingly, however, while the expression of *MAF1-4* is reduced by vernalization, the expression of *MAF5* is elevated, suggesting that MAF5 may serve a function opposite that of the other members of the clade (Ratcliffe et al. 2001 and 2003).

ENDOGENOUS MODULATION OF *FLC* EXPRESSION

FLC expression—and therefore flowering time—is also modulated by endogenous factors (Figure 1). FRIGIDA (FRI), a plant-specific coiled-coil domain protein, constitutively

upregulates *FLC* expression (Michaels and Amasino 1999; Johanson et al. 2000). The mechanism by which FRI upregulates *FLC* expression is not entirely clear, but FRI may affect *FLC* RNA processing via interaction with the nuclear cap binding complex, evidence for which stems in part from the fact that components of the nuclear cap binding complex are also required for FRI-mediated late flowering (Geraldo et al. 2009; Bezerra et al. 2004).

Several additional proteins also appear to act cooperatively with FRI in order to upregulate *FLC* expression. These include the FRI-related proteins FRIGIDA-LIKE 1 (FRL1) and FRIGIDA-LIKE 2 (FRL2), the function of either of which, but not both, is required for FRI-mediated upregulation of *FLC* expression (Michaels et al. 2004; Schläppi 2006). SUPPRESSOR OF FRIGIDA 4 (SUF4), another protein required for high *FLC* expression, has been shown to interact both with the *FLC* promoter and with FRI and FRL1 (Kim et al. 2006; Kim and Michaels 2006). Finally, FRIGIDA ESSENTIAL 1 (FES1) and FLC EXPRESSOR (FLX), two additional candidates for participation in a putative FRI complex, are also required for FRI-mediated *FLC* upregulation (Schmitz et al. 2005; Andersson et al. 2008).

FLC expression is also elevated by FRI-independent mechanisms, including by putative orthologs of the chromatin-remodeling SWR1 complex. The SWR1 complex has been most thoroughly characterized in yeast (Mizuguchi et al. 2004). In *Arabidopsis*, members of this SWR1 complex include PHOTOPERIOD INDEPENDENT EARLY FLOWERING 1 (PIE), ACTIN-RELATED PROTEIN6/SUPPRESSOR OF FRIGIDA3/ EARLY IN SHORT DAYS 1 (ARP6/SUF3/ESD1), and SERRATED LEAVES AND EARLY FLOWERING (SEF)/SWC6. The loss of members of this complex not only reduces *FLC* expression, but, as with the loss of members of the yeast SWR1 complex, also reduces the association of the histone variant H2A.Z with *FLC* chromatin, supporting a model in which these proteins, like their putative yeast

counterparts, catalyze H2A.Z deposition and thereby facilitate transcription (Noh and Amasino 2003; Choi et al. 2005; Deal et al. 2005; Martin-Trillo et al. 2006; March-Díaz et al. 2007; Lázaro et al. 2008).

Similarly, *FLC* expression is also elevated by putative orthologs of the PAF1 complex, which, in yeast, regulates transcription through the recruitment of an H3K4 methyltransferase and through association with RNA polymerase II (Krogan et al. 2002; Squazzo et al. 2002; Betz et al. 2002; Ng et al. 2003). In *Arabidopsis*, members of this PAF1 complex include VERNALIZATION INDEPENDENT 4 and 5 (VIP4/5), EARLY FLOWERING 7 (ELF7), and VERNALIZATION INDEPENDENT 6/EARLY FLOWERING 8 (VIP6/ELF8). The loss of members of this complex not only reduces *FLC* expression but leads also to loss of H3K4 methylation at *FLC* chromatin, supporting the idea that these *Arabidopsis* proteins facilitate transcription via mechanisms parallel to those of the yeast PAF1 complex (Zhang and van Nocker 2002; Oh et al. 2004; He et al. 2004).

Additional chromatin-modifying proteins also elevate *FLC* expression. EARLY FLOWERING IN SHORT DAYS/SET DOMAIN GROUP8 (EFS/SDG8), which is related to histone H3 methyltransferases that have been characterized in other systems, is required for high *FLC* expression and, in efs/sdg8 mutants, levels of H3K36 and H3K4 methylation are reduced at *FLC* chromatin (Kim et al. 2005; Zhao et al. 2005). Similarly, ARABIDOPSIS TRITHORAX 1 (ATX1) and ARABIDOPSIS TRITHORAX-RELATED 7 (ATXR7), which are related to yeast H3K4 methyltransferases, also elevate *FLC* expression and their loss is also accompanied by a loss of H3K4 methylation at *FLC* chromatin (Pien et al. 2008; Tamada et al. 2009). Additional proteins implicated in H2B monoubiquitination and deubiquitination, including HISTONE MONOUBIQUITINATION 1 and 2 (HUB1/2), UBIQUITIN-CONJUGATING ENZYME 1 and

2 (UBC1/2), and UBIQUITIN-SPECIFIC PROTEASE 26 (UBP26), also elevate *FLC* levels, again highlighting the importance of chromatin modification in maintaining high *FLC* expression (Cao et al. 2008; Gu et al. 2009; Xu et al. 2009).

FLC expression is also constitutively reduced by members of what is called "the autonomous pathway" (Simpson 2004). The autonomous pathway was first defined genetically. It has only subsequently become clear that the autonomous pathway does not describe a true pathway but is instead composed of members that, themselves, act mostly autonomously. Perhaps reflecting this independence of member function, the autonomous pathway consists both of predicted nucleic acid binding proteins and of predicted chromatin remodeling factors (Simpson 2004). Specifically, the autonomous pathway includes (1) LUMINIDEPENDENS (LD), a homeodomain containing protein, (2) FLOWERING LOCUS K HOMOLOGY DOMAIN (FLK), a putative RNA-binding protein with three KH domains, (3) FPA and (4) FCA, putative RNA-binding proteins that contain multiple RNA recognition motifs, (5) FY, an mRNA processing factor and FCA-interaction partner, (6) FVE, which is related to a component of a histone deacetylase complex characterized in mammals, and (7) FLOWERING LOCUS D (FLD), which is related to a histone demethylase characterized in mammals (Rédei 1962; Lee et al. 1994; Lim et al. 2004; Mockler et al. 2004; Koornneef et al. 1991; Schomburg et al. 2001; Macknight et al. 1997; Simpson et al. 2003; Quesada et al. 2003; Henderson et al. 2005; Ausín et al. 2004; Kim et al. 2004; Sanda and Amasino 1996; He et al. 2003; Shi et al. 2004). Underscoring the importance of the chromatin remodeling functions of FVE and FLD, several arginine methyltransferases that modify histones have also been shown to repress FLC expression (Wang et al. 2007; Schmitz et al. 2008; Niu et al. 2008). In addition, two relatives of FLD also repress *FLC* expression; however, in contrast to FLD itself and in contrast to the other

autonomous pathway members described above, the loss of these FLD relatives also delays flowering by an *FLC*-independent means (Jiang et al. 2007).

Although the effect of autonomous pathway members on flowering time is mediated entirely through the repression of *FLC* expression, it has been shown that members of the autonomous pathway serve additional functions unrelated to flowering time. It has been demonstrated that members of the autonomous pathway modulate the expression of many non-*FLC* genes and repress the expression of some transposable elements (Wilson et al. 2005; Marquardt et al. 2006; Bäurle et al. 2007; Bäurle and Dean 2008; Veley and Michaels 2008). Consistent with a role in many physiological processes, some double autonomous pathway mutants show dramatic phenotypes, including reduced fertility, reduced chlorophyll content and other abnormalities (Veley and Michaels 2008).

It has been suggested that the broader transcriptional and physiological effects of the autonomous pathway may stem from genome-wide roles in RNA-mediated silencing. Indeed, as described above, several members of the autonomous pathway are likely involved in RNA metabolism. That RNA silencing may also play a role in the regulation of *FLC* is underscored by the fact that *FLC* expression is also reduced by the activities of DICER-LIKE 1 and 3 (DCL1/3), which are involved in the generation of small RNAs (Schmitz et al. 2007). A report that a 3' region of *FLC* is targeted for repression by a small RNA also indicates that *FLC* may be subject to RNA-mediated silencing (Swiezewski et al. 2007).

NATURAL VARIATION IN FLOWERING TIME

The species *Arabidopsis thaliana* consists of many natural, genetically distinct populations, called accessions, that occupy a broad range of environments and differ in many

adaptive traits (Koornneef et al. 2004). Flowering time, measured as the total number of leaves formed up until the production of the first flower, also differs among these natural accessions. The extent of natural flowering time variation has been assayed under controlled laboratory conditions. These experiments have shown that some accessions, classified as early-flowering, flower with 10-20 leaves; others, classified as late-flowering, flower with over 100 leaves (Shindo et al. 2005; Werner et al. 2005a; Lempe et al. 2005). Other accessions flower with an intermediate number of leaves. Of course, since the total number of leaves at flowering differs, sometimes considerably, from experiment to experiment and from laboratory to laboratory, "early-flowering" and "late-flowering" are only relative terms and do not serve as an absolute classification. The use of these terms, and a broader characterization of a natural accession in terms of its flowering behavior, is often undertaken with reference to the flowering behavior of a laboratory control line, often Columbia (Col), Landsberg *erecta* (L*er*), Wassilewskija (Ws), or some artificial derivative of these three (Alonso-Blanco and Koornneef 2000). For general purposes, Col, which has a nonfunctional *FRI* allele and low *FLC* expression, may be defined as an early-flowering accession, and FRI-Col, an artificial line created by the introgression of a functional *FRI* allele from the San Feliu-2 (Sf-2) into a Col background, in which *FLC* is highly expressed, may be defined as a late-flowering accession (Johanson et al. 2000; Lee and Amasino 1995).

Because it is important to coordinate reproduction with favorable local conditions, it has been suggested that the flowering behavior of natural accessions may vary according to the local environments within which these accessions evolved. In support of this idea, studies using European accessions do show correlations between flowering time and latitude of origin, although these relationships are complex (Johanson et al. 2000; Stinchcombe et al. 2004 and

2005; Shindo et al. 2005; Lempe et al. 2005). These studies indicate that later flowering accessions more frequently originate from more northern regions, where winters are harsher and it may be particularly important to delay flowering until winter has passed. In more southern regions, where winters are milder, rapid cycling accessions are more common. Correlations between flowering time and water use efficiency have also been documented, and it is possible that selection for water use efficiency may also contribute to flowering time differences in these environments (McKay et al. 2003). It has also been suggested that, in some environments, flowering early, prior to late summer, may be a strategy by which to avoid the unfavorable conditions of late summer, such as excessive heat (Shindo et al. 2007).

These correlations between flowering time and environment, which are presumably the result of adaptation, may not be apparent in all of the environments from which *Arabidopsis* has been isolated. In some cases, this lack of correlation may be due to population history. For instance, accessions from North America show low genetic diversity and appear to be recent introductions from Europe, meaning that their flowering behavior may be the result of founder effects and may not reflect adaptation to the environments from which they were first collected (Zwan et al. 2000; Shindo et al. 2005). More ancient founder effects, such as the fact that regions of central and northern Europe appear to have been colonized by *Arabidopsis* only following environmental changes late in the last ice age, may also affect the extent to which flowering behavior is correlated with local environment (Sharbel et al. 2000).

Much of the research regarding the flowering behavior of natural accessions has been conducted under laboratory conditions, not under field conditions, and the extent to which these experiments may be relevant to flowering behavior in the wild remains unclear. A recent study aimed at addressing this question, which grew laboratory mutants at multiple field sites and in

multiple seasons, did confirm a role for FRI and for the photoperiod, vernalization, and autonomous pathways in flowering behavior in the wild (Wilczek et al. 2009). The study also showed that the degree to which mutations in these pathways affect flowering time differs according to season and field site, indicating that there are limited environmental windows within which these genes serve important roles. Attempts to address these questions in laboratory settings through the detailed simulation of wild conditions, as, for instance, a recent study which examined the behavior of lines in two growth chambers which simulated, respectively, Spain- and Sweden-like conditions, have also been initiated (Li et al. 2006).

Despite the large number of genes that have been shown to affect flowering time in artificially mutagenized populations, natural variation in flowering time has thus far been traced to allelic differences in only a few genes. In particular, natural alleles of the floral repressors *FRI* and *FLC* appear to be responsible for much of this variation. Loss of function mutations in *FRI* have arisen on at least ten independent occasions, and, in at least some of these cases, appear to have generated early-flowering accessions from late-flowering ancestors (Johanson et al. 2000; Le Corre et al. 2002; Gazzani et al. 2003; Lempe et al. 2005; Werner et al. 2005a; Shindo et al. 2005). A recent laboratory selection experiment employing mixed parent lines generated from 19 accessions supports the idea that allelic differences in *FRI* may underlie the evolution of early-flowering accessions (Scarcelli and Kover 2009). Finally, interestingly, allelic differences in *FRI* appear to contribute to natural variation not only in *A. thaliana* but also in the related species *A. lyrata* (Kuittinen et al. 2008).

Multiple independent loss of function *FLC* alleles have also been documented, which also appear to affect flowering time (Lempe et al. 2005; Werner et al. 2005a). Another class of natural *FLC* alleles that appear to affect flowering time are those that are weakly functional.

Most of the weak *FLC* alleles thus far documented contain transposable elements in the first intron (Michaels et al. 2003; Gazzani et al. 2003; Lempe et al. 2005). In one of these cases, it has been demonstrated that the transposon weakens *FLC* by rendering it the target of siRNA-mediated silencing (Liu et al. 2004).

Allelic differences in flowering time genes other than *FRI* and *FLC* also contribute to differences in the flowering behavior of natural accessions. Some of these genes regulate *FLC* expression. *FRIGIDA-LIKE 1* and *2* (*FRL1*/2), whose gene products act cooperatively with FRI to elevate *FLC* expression, differ among accessions: *FRL1* is functional and *FRL2* nonfunctional in the Col accession and the reverse is true in the L*er* accession (Michaels et al. 2004; Schläppi 2006). Similarly, a weak allele of *FY*, which encodes a member of the autonomous pathway, elevates *FLC* expression in the Bla-6 accession (Simpson 2004; Adams et al. 2009). Two rare alleles of *ENHANCER OF AG-4 2* (*HUA2*), which encodes a gene that plays a role in RNA processing and whose activity is required for high *FLC* expression, contribute to natural variation in flowering time (Chen and Meyerowitz 1999; Cheng et al. 2003; Doyle et al. 2005; Wang et al. 2007). In the L*er* accession, a weak *HUA2* allele reduces *FLC* expression; in the Sy-0 accession, a gain of function *HUA2* allele elevates *FLC* expression with reference to control accessions (Doyle et al. 2005; Wang et al. 2007).

FLOWERING LOCUS M/*MADS AFFECTING FLOWERING 1* (*FLM*/*MAF1*), an *FLC* relative that appears to delay flowering by the same mechanisms as *FLC*, also contributes to natural flowering time variation: in the accession Nd-1, a large scale deletion removing the entire *FLM* gene accelerates flowering time (Scortecci et al. 2001; Ratcliffe et al. 2001; Werner et al. 2005b). Downstream of *FLM* and *FLC*, differences in the cis regulatory regions of the gene

encoding the floral integrator *FT* contribute to differences in the flowering time of the accessions Est-1 and Col (Schwartz et al. 2009).

Variant alleles of genes encoding photoreceptors also contribute to natural variation in flowering time. In the Cvi accession, a more stable variant of CRYPTOCHROME (CRY2) alters day length sensitivity and accelerates flowering under short day conditions (El-Assal et al. 2001). In the Fr-2 accession, a premature stop codon in *PHYTOCHROME C* (*PHYC*) causes early flowering in short days (Balasubramanian et al. 2006). Meanwhile, many *Arabidopsis* accessions can be classified as possessing either a strong Col-type *PHYC* allele or a weak L*er*-type *PHYC* allele. The possession of these alleles appears to vary on a latitudinal cline, with the strong Col-type allele predominating in more northern regions, where, due to harsher winters, an enhanced ability to distinguish long days and short days may be more critical (Balasubramanian et al. 2006). Finally, *PHYTOCHROME D* (*PHYD*) also contributes to flowering time variation: a 14 bp deletion in *PHYD* accelerates flowering in the Ws accession (Aukerman et al. 1997).

Although several genes underlying natural variation in flowering time have been identified, there remain many accessions that exhibit flowering behaviors for which the molecular explanations remain unknown. In some accessions, there appears to be no correlation between flowering time and *FLC* expression: some accessions are early-flowering despite relatively high *FLC* expression and others are late-flowering despite low *FLC* expression (Shindo et al. 2005). More strikingly, the accession Bur-0, despite possessing an apparent null *FLC* allele, is nonetheless late-flowering (Werner et al. 2005a). Other accessions do not seem to require *FRI* for high *FLC* expression: the accession Lz-0, despite possessing a nonfunctional *FRI*, expresses *FLC* at high levels and is late-flowering (Shindo et al. 2005; Werner et al. 2005a). Still other accessions are "vernalization insensitive," i.e., in these accessions, flowering is not

accelerated by prolonged cold treatment (Karlsson et al. 1993; Shindo et al. 2005 and 2006; Werner et al. 2005a; Lempe et al. 2005). The genomic differences responsible for these novel flower behaviors remain unknown.

Many approaches have been employed to define quantitative trait loci (QTLs) responsible for natural variation in flowering behavior; some of these approaches have also been used to identify causative genes. Most of these approaches entail crossing two accessions with different flowering phenotypes to create mixed parent populations that segregate for the parental phenotypes. Sometimes, mixed parent populations exhibit phenotypes not found in the parent populations, a phenomenon called transgression. Using genome-wide molecular markers, individual plants—sometimes all members of the population, sometimes only those that exhibit the most extreme phenotypes—are genotyped. Many polymorphic markers with which to distinguish genomic regions from different natural accessions have been identified; the range of these markers is perhaps best illustrated by their recent use to propose phylogenetic relationships among hundreds of natural accessions (Nordborg et al. 2005; El-Lithy et al. 2006; Schmid et al. 2006; Clark et al. 2007). After individual plants are genotyped, an attempt is made to correlate genotypes with flowering behaviors; such correlations, if they exist, may define genomic regions responsible for the phenotypes. The same process, continued at a finer scale, may also be used to identify causative genes.

In these simple F2 populations, the identification of causative regions may be complicated the presence of multiple contributing loci, which may participate in complex interactions, such that the effects of some loci are obscured. One way of addressing this problem, and of examining the effects of only one genomic region at a time, is to construct near-isogenic lines (NILs). NILs are constructed by introgressing a selected region from one parent

into the genetic background of a second parent. FRI-Col, a widely used laboratory strain, is itself a NIL, and consists of the functional *FRI* allele from Sf-2 introgressed into a Col background (Lee and Amasino 1995). Multiple whole genome NIL libraries have been constructed for use, in part, in the dissection of flowering phenotypes (Keurentjes et al. 2007; Törjék et al. 2008). Individual NILs can also be combined genetically in order to examine interactions between multiple loci (Eshed and Zamir 1996; Reif et al. 2009).

In order to introgress a small region from one parent (minority parent) into the background of a second parent (majority parent), two parents are crossed to create an F1, a line consisting 50% of each parent. The F1 is crossed again to the majority parent, a step called the backcross 1, creating a backcross 1 plant consisting 25% of the minority parent and 75% of the majority parent. A backcross 1 plant that has retained the desired region from the minority parent is selected and then crossed again to the majority parent in order to create subsequent backcross generations. At each generation, the fraction of nuclear DNA contributed by the minority parent can be calculated using the formula $1/2^{(N+1)}$, in which N is the number of backcrosses. Therefore, at the backcross 3 generation, 6.25% of the line will be from the minority parent and at the backcross 6 generation, less than 1% of the line will be from the minority parent. It is to be emphasized that this formula assumes random segregation and therefore refers only to those parts of the genome that are outside of and unlinked to the region that is specifically retained at each stage of the backcross. In fact, the region selected often consists of multiple Mb, such that, at late stages of the backcross, the selected region itself constitutes a greater percentage of minority parent DNA than predicted by the formula. The formula also does not account for the non-random segregation, as might occur, for instance, with the preferential survival of plants carrying an additional minority parent locus. Indeed,

segregation distortion (SD) has been observed in many mixed parent populations (Törjék et al. 2006; Simon et al. 2008). The molecular basis of some of the more severe cases of SD, in which the combination of two alleles leads to severe necrosis or is lethal, has been at least partially elucidated: SD appears to stem, in one case, from an autoimmune response, and, in a second case, from the loss of functional variants of a gene required for histidine biosynthesis (Bomblies et al. 2007; Bikard et al. 2009). The molecular basis of more subtle instances of SD, which may also affect the construction of mixed parent lines, remains unknown.

Although the NIL has the advantage of enabling the examination of the effects of a single genomic region in isolation, without the possible confounding effects of multiple modifier loci, it may not be appropriate for all purposes. Because a NIL requires molecular selection at each generation, its construction is laborious. In addition, the effects of some loci may not be visible in certain NILs due to the very fact that modifier loci are not present. An alternative approach that addresses these potential problems is the recombinant inbred line (RIL) population (Shindo et al. 2007). A RIL population is generated by selfing offspring from a simple F2 for many generations, in order to create many homozygous lines, each fixed for a unique genetic combination from the two parents. RILs are not genotyped during their construction, only following their completion, and are therefore less laborious to construct. Many RIL populations have been generated, for use, in part, in the dissection of flowering phenotypes (Lister and Dean 1993; Alonso-Blanco et al. 1998; Loudet et al. 2002; El-Lithy et al. 2004 and 2006).

NILs and RILs have two major advantages with reference to standard F2 populations. In both NILs and RILs, population heterozygosity is substantially reduced; because many loci act semidominantly, heterozygosity is a potentially confounding factor in F2 populations. In addition, because both NILs and RILs are genetically fixed, these lines also have the advantage

that they can be compared across repeated experiments. For instance, the same lines can be used to examine flowering behavior under multiple environmental conditions, as, for example, before and after vernalization. Of course, NILs and RILs represent distinct approaches: a study comparing the utility of NILs and RILs concluded that NILs can be used to detect smaller effect loci than RILs, but that the resolution power of RILs is higher (Keurentjes et al. 2007).

A variant on the RIL population is the Advanced Intercross RIL (AI-RIL) population. To generate an AI-RIL population, offspring plants are repeatedly crossed to one another over several generations prior to several generations of selfing. These multiple intercrosses increase the number of recombination events per line and thereby increase locus resolution (Balasubramanian et al. 2009). Yet another variant of this approach is called Multiparent Advanced Generation Inter-Cross (MAGIC). This approach is similar to the production of AI-RILs, with the exception that, in MAGIC, multiple accessions (in a recent project, 19 *Arabidopsis* accessions) serve as the parents of a single population (Kover et al. 2009). MAGIC populations enable the examination of larger numbers of QTL and can be used to address broader, species-wide questions.

Another approach, association mapping (also known as linkage disequilibrium (LD) mapping) is distinct from other mapping approaches in that it does not involve generating new offspring lines but instead aims to correlate phenotypes with causative loci through analyses of the natural accessions themselves (Flint-Garcia et al. 2003; Shindo et al. 2007). A major obstacle to the use of association mapping in *Arabidopsis* is the existence of population structure, which, if it is not appropriately controlled for, can produce false positives; however, ways to minimize the effects of population structure have been proposed (Hagenblad et al. 2004; Aranzana et al. 2005; Zhao et al. 2007; Ehrenreich et al. 2009).

There are increasing indications that epigenetic differences may make important contributions to natural variation, at least genomically, and, in mapping causative loci, it may also be important to consider epigenetic effects (Vaughn et al. 2007; Zhai et al. 2008). These considerations present unique challenges; for instance, it has been demonstrated that, in mixed-parent populations, epi-alleles may become unstable, likely rendering them difficult to map (Johannes et al. 2009; Reinders et al. 2009; Reinders and Paszkowski 2009).

It has already been demonstrated that epigenetic differences contribute to natural variation in flowering time. Epigenetic silencing, induced by the presence of a transposable element in the first intron of L*er FLC*, is responsible for the weakness of the L*er FLC* allele and contributes to the early-flowering phenotype of the L*er* accession (Liu et al. 2004). In a broader sense, the importance of chromatin modifications both in the expression of *FLC* and in *FLC* repression following vernalization is consistent with the possibility that *FLC*-centered epigenetic mechanisms might contribute to flowering-time variation in additional instances (He and Amasino 2005). This possibility is particularly underscored by the fact that *FLC* expression is affected both by DICER-LIKE 1 and 3 (DCL1/3), which are involved in the generation of small RNAs, and by a siRNA directed at a 3' region of *FLC* (Schmitz et al. 2007; Swiezewski et al. 2007). In light of these data, models in which differences in cis elements within natural alleles of *FLC* might affect the recruitment of activating or silencing factors and thereby affect *FLC* expression might be suggested. Possibly consistent with such a model is the fact that, in at least some vernalization insensitive accessions, in which *FLC* is not stably silenced, vernalization insensitivity appears, in part, to map to the top of Chromosome V, to the region within which *FLC* itself is located (Shindo et al. 2006).

At least some of the epigenetic differences that distinguish natural accessions are due not to the affected loci themselves but to differences in transacting factors; in light of these examples, models in which variation in unlinked modifier loci might affect the epigenetic state of *FLC* and thus affect flowering time might also be proposed. For instance, it has been shown that a region adjacent to the *FLC* promoter, whose sequence is identical in L*er* and Col, is methylated in L*er* but not in Col, indicating differences in the activity of transacting factors (Zhai et al. 2008). It is important, however, to note that the methylation of this region does not appear to affect *FLC* expression or flowering time. In addition, in the Bor-4 accession, a large deletion eliminates function of *VARIANT IN METHYLATION 1*/*ORTHRUS 2* (*VIM1*/*ORTH2*), which encodes a methylcytosine-binding protein. Loss of VIM1/ORTH2 function causes hypomethylation of Bor-4 centromeric DNA (Woo et al. 2007). This example indicates that variation in the function of the epigenetic machinery is tolerated in natural populations. Although loss of *VIM1* alone does not alter flowering time, the loss of *VIM1* together with the loss of two *VIM1* relatives delays flowering time by altering the expression of unlinked genes (Woo et al. 2008). Interestingly, overexpression of *VIM1* or of one of its close relatives also delays flowering, seemingly by the same mechanism (Kraft et al. 2008). Whether these or parallel mechanisms may contribute to differences in the flowering behavior of natural populations—and whether the operation of such mechanisms may complicate the mapping of flowering-time loci—remains to be determined.

TUL-0: A NATURAL ACCESSION WITH TWO UNUSUAL FLOWERING PHENOTYPES

In this work, we examine Tul-0, a natural *Arabidopsis* accession, first collected in the mid-western United States, that exhibits two unusual flowering phenotypes. First, despite possessing a weak allele of *FLC*, Tul-0 is late-flowering. Second, Tul-0 exhibits a reduced response to vernalization, flowering late both before and after extended cold treatment.

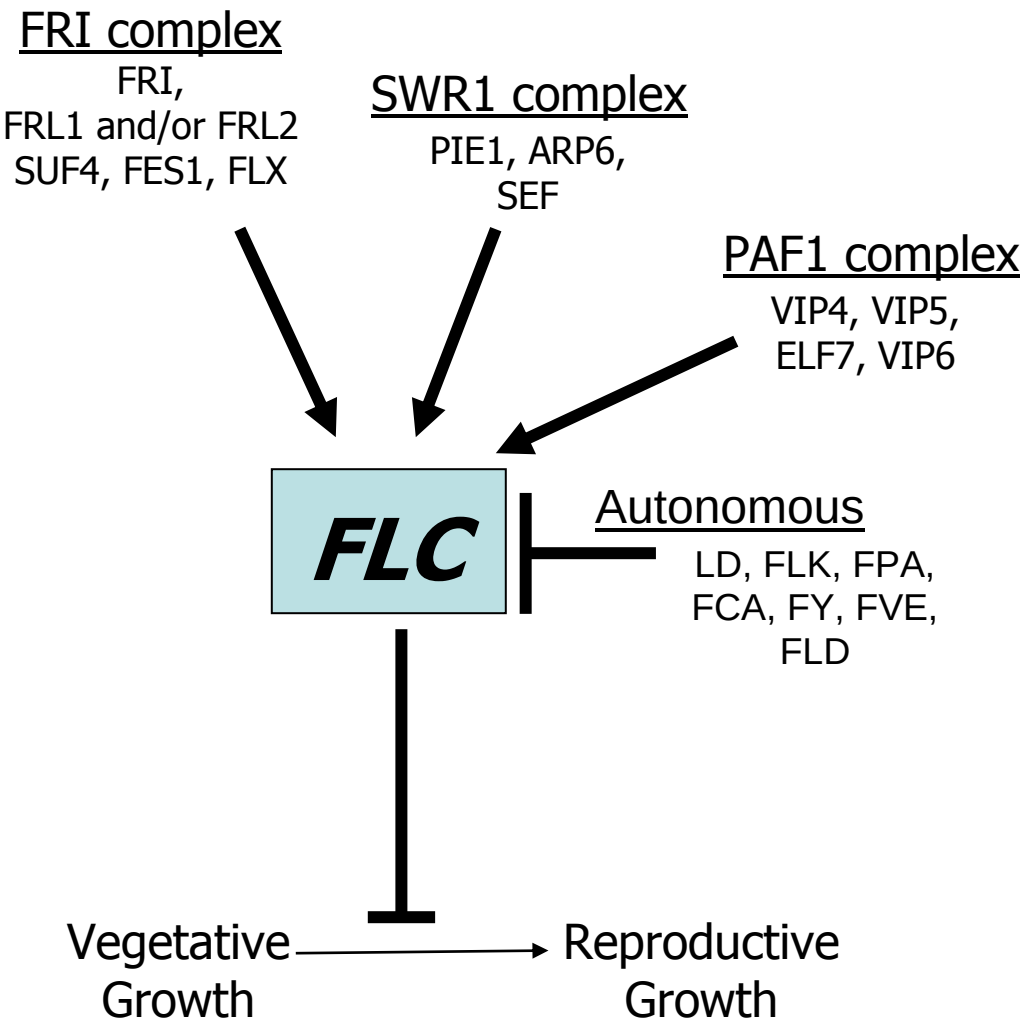
In chapter 2, we characterize both phenotypes, showing, first, that Tul-0 remains late-flowering following 1-2 months of vernalization. We also show that, in mixed-parent populations produced by crossing Tul-0 to the late-flowering laboratory accession FRI-Col, some members are early-flowering, a phenotype not displayed by either parent. We map this phenotype to Tul-0 *FLC*, which, like many weak *FLC* alleles, contains a transposable element in the first intron. We also present evidence that Tul-0 belongs to a clade of North American accessions, all of which contain the same *FLC* allele and many of which exhibit the same behavior following vernalization. Finally, we further characterize the Tul-0 *FLC* allele, showing that it is responsive to *FRI* and to the loss of some, but not all, members of the autonomous pathway.

In chapter 3, we begin to dissect the genetic basis for the fact that Tul-0 is late-flowering despite possessing a weak *FLC* allele. We define two Tul-0 loci that compensate for the weak *FLC*. The first, *LATE INDEPENDENT of FLC* (*LIF*), is located at the top of Chromosome V and delays flowering via an *FLC*-independent mechanism. The second, *FLC-ENHANCER 1* (*FEN1*), is located at the bottom of Chromosome I and acts via an *FLC*-dependent mechanism. We also present evidence that additional *FEN* loci also contribute to the late-flowering phenotype. We show that the action of the *FEN* loci is accompanied by an upregulation of *FLC*

expression, and is in part *FRI*-independent. In addition, we show that these *FEN* loci do not act exclusively on the weak Tul-0 *FLC* allele but also enhance the weak Ler and Da (1)-12 *FLC* alleles, as well as the strong Col *FLC* allele.

In chapter 4, we examine the molecular basis for Tul-0's reduced vernalization sensitivity. Mapping lines indicate that this reduced sensitivity is caused by at least two Chromosome V loci–one which may be *FLC* itself, and the other, *REDUCED VERNALIZATION RESPONSE1* (*RVR1*), that may be distinct from both *FLC* and *LIF*. *RVR2*, a locus in the middle of Chromosome I, also appears to contribute to this phenotype. Finally, we show that, as in FRI-Col, Tul-0 *VIN3* is elevated by vernalization. However, in contrast to FRI Col, in which *FLC* expression is permanently reduced by vernalization, in Tul-0, *FLC* expression is initially reduced by cold treatment, but this reduction is not stable and expression again increases following a return to warm conditions.

Figure 1. Endogenous modulation of *FLC* expression. Levels of *FLC*, a floral repressor, are elevated by members of the FRI, PAF1, and SWR1 complexes, and repressed by members of the autonomous pathway.


FRI complex
FRI,
FRL1 and/or FRL2
SUF4, FES1, FLX
SWR1 complex
PIE1, ARP6,
SEF
PAF1 complex
VIP4, VIP5,
ELF7, VIP6
FLC
Autonomous
LD, FLK, FPA,
FCA, FY, FVE,
FLD
Vegetative
Growth
Reproductive
Growth

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CHAPTER 2

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF THE TUL-0 ACCESSION

ABSTRACT

The initiation of reproductive development represents an important developmental transition in the plant life cycle. In *Arabidopsis*, the timing of this transition is in part determined by the negative floral regulator FLOWERING LOCUS C (FLC). FLC levels are reduced both by the experience of prolonged winter cold, during a process called vernalization, and by endogenous factors, including members of the autonomous pathway. Natural populations of *Arabidopsis* exhibit considerable variation in the timing of the flowering transition, and much of this variation can be traced to differences in *FLC* expression. Here, we examine Tul-0, a late-flowering *Arabidopsis* accession that exhibits a reduced sensitivity to vernalization. We show that, in mixed-parent populations produced by crossing Tul-0 to the late-flowering laboratory accession FRI-Col, some members are early-flowering, a phenotype not displayed by either parent. We map this phenotype to Tul-0 *FLC*, which, like many weak *FLC* alleles, contains a transposable element in the first intron. We consider this transposon with reference to a related transposon in the *FLC* allele of a different accession and with reference to the three most closely related transposons in the Col genome. We also further characterize the Tul-0 *FLC* allele, showing that, although it is weak, it is functional. At the same time, we document the curious fact that Tul-0 *FLC* lines are responsive to some but not all members of the autonomous pathway.

INTRODUCTION

In *Arabidopsis*, as in many plant species, the timing of flower initiation is governed by many factors, both environmental and endogenous. Day length, which is perceived in leaves, serves as an important seasonal cue (Turck et al. 2008). Under short day conditions, flowering is

inhibited and, under long day conditions, flowering is accelerated. Another important seasonal cue is temperature. In many environments, a period of prolonged cold—i.e., winter—serves as a reliable indicator that spring is imminent. During a process called vernalization, the experience of prolonged cold accelerates the plant's capacity to flower (Chouard 1960). This requirement for prolonged cold distinguishes vernalization from short-term cold responses such as cold shock or seed stratification.

Vernalization accelerates flowering by reducing expression of the negative floral regulator *FLOWERING LOCUS C* (*FLC*) (Sheldon et al. 1999; Michaels and Amasino 1999). The vernalization-mediated reduction of *FLC* expression involves the modification of *FLC* chromatin and appears to be at least in part dependent upon the formation of a Polycomb Repressive Complex 2 (PRC2)-like complex, in association with several plant homeodomain (PHD) proteins (Bastow et al. 2004; Sung and Amasino 2004; Wood et al. 2006; De Lucia et al. 2008).

FLC expression is also modulated by endogenous factors, which also affect flowering time. *FLC* expression is constitutively reduced by members of the autonomous pathway, which include FCA, FLOWERING LOCUS K HOMOLOGY DOMAIN (FLK), FPA, FLOWERING LOCUS D (FLD), FVE, and LUMINIDEPENS (LD), and whose respective functions are predicted to include RNA processing and chromatin modification (Simpson 2004). Conversely, *FLC* expression is constitutively elevated by the plant-specific protein FRIGIDA (FRI) and by plant homologs of the PAF1 and SWR1 complexes from yeast (Michaels 2008).

Many of the genes shown to affect floral timing were first identified through work with mutagenized populations. However, natural populations—or accessions—of *Arabidopsis*, which exhibit extensive variation in flowering behavior, have served as another powerful tool. When

quantified under laboratory conditions in terms of the number of leaves formed at flowering, the earliest-flowering accessions flower with fewer than 10 leaves and the latest-flowering accessions flower with more than 100 leaves. Natural accessions also show different sensitivities to day length and to vernalizing cold treatment (Shindo et al. 2005 and 2006; Werner et al. 2005; Lempe et al. 2005; Giakountis et al. 2009). The loci underlying these flowering behaviors can be mapped using standard techniques or any of many derivations thereof (Shindo et al. 2007).

Preliminary analyses have shown that many accessions are early-flowering due to the possession of a null allele of *FRI* or *FLC* (Johanson et al. 2000; Le Corre et al. 2002; Gazzani et al. 2003; Shindo et al. 2005; Lempe et al. 2005; Werner et al. 2005). Weak but functional *FLC* alleles, which delay flowering to a limited extent but cannot confer a late-flowering phenotype, also contribute to natural variation in flowering behavior. Many of these weak *FLC* alleles, including those from the L*er*, Kin-0, Da (1)-12 accessions, contain transposable elements in the first intron (Gazzani et al. 2003; Lempe et al. 2005; Michaels et al. 2003). In the case of L*er*, it has been demonstrated that the transposon itself is responsible for the weakness of L*er FLC* (Michaels et al. 2003; Liu et al. 2004).

There are also many accessions in which flowering time is not proportional *FLC* expression. Some accessions are late-flowering despite low *FLC* expression, others are early-flowering despite high *FLC* expression, and the molecular basis of these behaviors remains unclear (Shindo et al. 2005). Other accessions show a reduced sensitivity to vernalization, and, despite some preliminary investigations, the molecular basis of this reduced sensitivity also remains poorly characterized (Shindo et al. 2006).

Here, we begin work with Tul-0, a late-flowering North American accession that exhibits a reduced sensitivity to vernalization. In efforts to map loci required for vernalization

insensitivity, we reveal a second phenotype: in mapping populations, some plants are early-flowering even in the absence of vernalization. We map this phenotype to Tul-0 *FLC* and find that Tul-0 *FLC*, like many weak *FLC* alleles, contains a transposable element in the first intron. Finally, we show that, although Tul-0 *FLC* is functional and responsive to *FRI*, it exhibits atypical responses to some members of the autonomous pathway.

MATERIALS AND METHODS

Plant materials:

Tul-0 was first collected in 1985 from a site just north of Turk Lake, Michigan, by Fredric Lehle, then associated with the MSU-DOE Plant Research Laboratory. Tul-0 has been described previously (Karlsson et al. 1993). Yo-0, Rmx-A02, Pna-10, Knox-10, RRS-10, Knox-18, Pna-17 seeds were provided by Todd Michael and Julin Maloof. These accessions have also been described previously (Shindo et al. 2005).

FRI-Col was created by the introgression of an active *FRI* locus from the accession San Feliu-2 (Sf-2) into the Col genetic background and has been previously described (Lee and Amasino 1995). The L*er* accession and the transposon-containing L*er FLC* allele have also been previously described (Gazzani et al. 2003; Michaels et al. 2003; Liu et al. 2004).

The null *fri* allele from Col has been previously described (Johanson et al. 2000). The autonomous pathway mutations (Col background) used to create the Tul-0 *FLC* and L*er FLC* autonomous pathway lines have been previously described: *fpa-7* (Michaels and Amasino 2001), *fca-9* (Bäurle and Dean 2008), *ld-1* (Rédei 1962), *fld-3* (He et al. 2003), and *fve-4* (Ausín et al. 2004). *flk* (SALK_112850) has been described, variously, as *flk-1* (Lim et al. 2004) and *flk-4* (Mockler et al. 2004).

Line Construction:

Lines in a Tul-0 background were constructed through continued backcrossing, employing Tul-0 as the recurrent maternal parent, whilst selecting for the retention of a given region from the minority parent at each generation. Construction of lines in a FRI-Col background was the same, save that FRI-Col was the recurrent maternal parent. Similarly, L*er* served as the recurrent female in the construction of lines in a L*er* background. In these introgression lines, the fraction of nuclear DNA contributed by the minority parent can be estimated using the formula $1/2^{(N+1)}$, in which N is the number of backcrosses. Flowering of intermediate backcross generations was accelerated by continuous growth under far red light and twice weekly treatment with a 0.04 g/L GA, 5% ethanol solution.

Line description:

Tul-0 *FLC* FRI-Col was created by introgressing Tul-0 *FLC* into a FRI-Col background out to the backcross 9 generation. A recombinant line was selected specifically to exclude Tul-0 genomic material beneath 4.6 Mb on ChromosomeV, a region physically linked to *FLC*.

Tul-0 *FLC* L*er* was created by introgressing Tul-0 *FLC* into a L*er* background out to the backcross 5 generation. A recombinant line was selected specifically to exclude Tul-0 genomic material beneath 4.6 Mb on Chromosome V, a region physically linked to *FLC*.

L*er FLC* FRI-Col was created by introgressing L*er FLC* into a FRI-Col background out to the backcross 5 generation. A recombinant line was selected specifically to exclude the nonfunctional L*er* allele of the flowering time gene *FRIGIDA LIKE 1* (*FRL1*), which is physically linked to *FLC* on Chromosome V (Schläppi 2006).

fri Tul-0 *FLC* Col was created by crossing Tul-0 *FLC* FRI-Col to Col, which carries a nonfunctional *fri* allele. *fri/fri* Tul-0/Tul-0 *FLC* was selected in the F2 generation. *fri* L*er FLC* Col was created by crossing L*er FLC* FRI-Col to Col, using a parallel approach.

Tul-0 *FLC* FRI-Col *fca*, *flk*, *fpa*, *fld*, *fve*, and *ld* lines were created by crossing Tul-0 *FLC* FRI-Col (described above) to *FRI fca*, *FRI flk*, *FRI fpa*, *FRI fld*, *FRI fve*, and *FRI ld* mutants and selecting for the desired combinations in the F2, or if necessary, the F3 generations. L*er FLC* FRI-Col *fca*, *flk*, *fpa*, *fld*, *fve*, and *ld* lines were created through a parallel approach.

Tul-0 *FLC* (#2) FRI-Col is distinct from Tul-0 *FLC* FRI-Col; it was created by an independent introgression of Tul-0 *FLC* into a FRI-Col background out to the backcross 5 generation. Tul-0 *FLC* (#2) FRI-Col is also distinct from Tul-0 *FLC* FRI-Col in that it retains Tul-0 genomic regions beneath 4.6 Mb on Chromosome V linked to *FLC*.

fri Tul-0 *FLC* (#2) Col *fca*, *flk*, *fld*, *fve*, and *ld* lines were created by crossing Tul-0 *FLC* (#2) FRI-Col to (*fri*) Col, *fri fca*, *fri flk*, *fri fld*, *fri fve*, and *fri ld* and selecting for the desired combinations in the F2, or if necessary, the F3 generations.

Description of segregating populations used to generate figures:

Segregating Col/Tul-0 for *FLC* in a FRI-Col background (Figure 2a): Tul-0 *FLC* FRI-Col (described above) was crossed to FRI-Col to generate a backcross 10 F1, which served as the parent of this population.

Segregating for Tul-0/L*er* for *FLC* in a FRI-Col background (Figure 4b): Tul-0 *FLC* FRI-Col and L*er FLC* FRI-Col (both described above) were crossed to create an F1, which served as the parent of this population.

Growth conditions:

Seeds were sterilized with a 70% ethanol 0.1% Triton X-100 solution, then transferred to plates containing 0.65 g/L Peters Excel 15-5-15 fertilizer (Grace Sierra) within a solid agar medium. On plates, seeds were incubated for 1 week in the dark at 4°C, then transferred to continuous light for 3 days to enable germination. For vernalization experiments, plates were subsequently transferred to a 4°C chamber under cool-white fluorescent lights under short day conditions (8 hours light/16 hours dark) for the specified vernalization period. Following the cold treatment, plants were transferred to soil and grown at 22°C under cool-white fluorescent lights under long day conditions (16 hours light/8 hours dark). For non-vernalization experiments, plants were transferred directly to soil following 3 days of germination.

For the vernalization time courses, treatment regimens were staggered such that all plants within a given experiment were transferred to soil on the same day.

Flowering time measurements:

Flowering time was measured as the total number of leaves (rosette + cauline, cotyledons omitted) formed at the time of the appearance of the first flower. Following 3 weeks of growth on soil, leaves were counted weekly. Each counted leaf was marked with a fine point black permanent marker (Sharpie) in order to avoid multiple counts of the same leaf.

The 0-120 day vernalization time course was terminated after 4 months of growth on soil, before the latest subset had flowered. Plants that had not flowered within this period were simply assigned a value of 120 total leaves.

PCR primers:

Col vs. Tul-0 mapping primers: Indels flanked by the following primers sets were used to genotype the earliest flowering members of a Tul-0/FRI-Col backcross 3 population: On Chromosome I: 3 Mb: acccaagtgatcgccacc and aaccaaggcacagaagcg; 12.2 Mb: ctcttttctattagaaccaatgggag and aacttaaatatgagaaaacacacaatgc; 21.2 Mb: tgtccaaattctctacctctgctg and aaagctatataaagaaagagctgg; 30 Mb: ctctttatgggtttgcctc and gtgtccaggtggcattat. On Chromosome II: 5.3 Mb: acatgaacgatctaaggtctaatgt and gcatggctgatcactggtgacttca; 12 Mb: gcattactccggtgtcgtc and gaatctcaatatgtgtcaac; 16.2 Mb: gtattgatatccagtcacattacta and caataacttgatggtctaatgactg. On Chromosome III: 8.9 Mb: ggattaaggattcaagtgagacaac and ctccacttcacctttatcatcatca; 16.8 Mb: tcatctctcttaggcattttcacaa and gctcctttacctaacccaactgcat. On Chromosome IV: 8.8 Mb: gcttgctctcaacgcttaagccact and ataaacttggtttaggagctacgat; 17.3 Mb: ccttcctgaatcagaaattcaagaa and tcctcgttattctgcggtaagtgaa. On Chromosome V: 2.2 Mb: gaggacaaattacatatcttcata and cgaagagagtttgtaggagaaggg; 6.8 Mb: cgtcatacccatcatcacattcacc and cctctcagcaccgatctggataagc; 7.7 Mb: gggaaattcacgtttttctccagc and aaataacggaagttaagctgacgg; 15.3 Mb: caggcaccatctgtgttgcatttc and ggaatggaataaccgcaaggaacc; 24.1 Mb: tcactgaggcaactgtttgcgcctt and acgacacaattagttcagacacata.

FLC: A 30 bp indel in the first intron was used to distinguish Col *FLC* (+) from Tul-0 *FLC* (-) and L*er FLC* (-), using primers ctagaggcaccaaagaaacaaggc and tgttcctattcgttaaaattgaca. Tul-0 *FLC* and L*er FLC* were distinguished using a transposon-specific primer, atcgcttctagatctaagaatgatccc, and the reverse-oriented *FLC* primer, gtgggaaactataaacctttggac, which directs amplification of a region of Tul-0 FLC, and the forward-oriented *FLC* primer, aactttatctgtatgcctttgtatgac, which directs amplification of a region of L*er FLC*.

4.6 Mb Chromosome V primers: An indel at 4.6 Mb on Chromosome V, flanked by primers ttttgggaagttttgctggaatag and gtacagtctaaaagcgagagtatg, was used to select recombinants during the construction of the some of the introgression lines.

FRL1 primers: dCAPs markers to distinguish the nonfunctional L*er FRL1* from the functional Col *FRL1* have been previously described (Schläppi 2006).

FRI: The functional SF-2 FRI allele was distinguished from the nonfunctional Col allele by means of a 16 bp deletion flanked by the primers agtggttcgaatgagattgccgg and gaacttgatgttggtcgatgatg.

Autonomous pathway: *ld-1*, CAPS marker, with MseI: gaatatcttcctgttacgacacg and gctgcgtagctttcatcaatgcca; *fve-4*, dCAPS marker, with DdeI: caatatgttgatttcaggttctcactt and ctcagaagtggacataccaaatc; *fca-9*, dCAPS marker, with AluI: taccatgagagtaagctgtgaatcac and ccgaacaatcaatggctgattgcaag; *fld-3*, T-DNA allele with LB primer, tcagggacatttattccttgaggttca and caagatgacccatatgatatgggtggaga amplify WT; *fpa-7*, T-DNA allele with LB primer, aaggatatgatcctgctcagttggt and aaatcttcttccctgtctggtacga amplify WT; *flk*, T-DNA allele with LB primer, tgatctaaagttttcaggcactagc and ccttgccatctggctttggttcttcatatt amplify WT.

Real-Time: (*FLC*): tccggcaagctctacagcttc and agcatgctgtttcccatatcgatc; *UBIQUITIN* (*UBQ*): ctaccgtgatcaagatgcagatc and ttgtcgatggtgtcggagctttc

Sequencing:

Sequencing of Tul-0 *FLC* was performed at the Biotechnology Center at the University of Wisconsin-Madison. Sequencing of accessions using a pre-defined set of polymorphic markers (Clark et al. 2007) was performed at the University of Chicago.

Expression analysis:

Seeds were plated and germinated as described above. Entire seedlings were harvested. Tissue was frozen in liquid nitrogen, and total RNA was isolated using TRIzol reagent (Invitrogen), following the manufacturer's protocol, and resuspended in 30uL of DEPC-treated water. From this total RNA, 3 uL were used to synthesize first-strand cDNA employing M-MLV Reverse Transcriptase (Promega), following the manufacturer's protocol. Real-time PCR was performed on the 7500 Fast Real-time PCR system (Applied Biosystems) using DyNAmo HS SYBR Green qPCR kit (Finnzymes), following the manufacturer's protocol. The following PCR conditions were employed: one cycle of 15 min at 95°C and 40 cycles of 15 s at 95°C, 15 s at 60°C, and 30 s at 72°C, followed by a dissociation stage, according to the manufacturer's recommendations. *FLC* and *UBQ* primers are described above. Real-Time graphs shown in this manuscript are the averages of three technical replicates, with *FLC* values normalized to *UBQ*. These results in turn were consistent with at least two additional biological replicates.

RESULTS

Tul-0 exhibits a reduced sensitivity to vernalization

We selected Tul-0, a natural accession first collected in Michigan, USA, as the subject of our study. Tul-0 has been previously reported to exhibit a late-flowering phenotype both before and after a 24-day vernalization treatment (Karlsson et al. 1993). To further characterize this behavior, we subjected Tul-0 to a 6-month vernalization time course, employing the laboratory strain FRI-Col as a vernalization-sensitive control (Lee and Amasino 1995) (Figure 1). Tul-0 remained very late-flowering following 1 month of cold exposure; however, reminiscent of work with a collection of Swedish accessions (Shindo et al. 2006), this lateness was steadily reduced

by additional months of treatment, and, by six months, Tul-0 flowered as early as the FRI-Col control (Figure 1).

A transgressive early-flowering phenotype maps to Tul-0 *FLC*

In order to map the loci responsible for delayed flowering following 1-2 months of cold exposure, we crossed Tul-0 to FRI-Col, and, over several generations, backcrossed vernalization-insensitive offspring to the FRI-Col parent. At the backcross 3 generation, we noted that some members of our segregating F2 populations were early-flowering even in the absence of vernalization, a phenotype not exhibited by either parent. We genotyped these early plants at multiple genome-distributed markers and found that all of them were homozygous Tul-0 at the top of the Chromosome V, in a region containing *FLC*.

We continued to introgress Tul-0 *FLC* into a FRI-Col background out to the backcross 9 generation, creating an early-flowering Tul-0 *FLC* FRI-Col line. In order to verify that, following this advanced introgression, the phenotype continued to segregate with Tul-0 *FLC*, we crossed Tul-0 *FLC* FRI-Col again to FRI-Col, creating a backcross 10 F1. We planted the offspring of this F1 and for each plant determined both the total leaf number at flowering and the genotype at *FLC* (Figure 2a). In this population, Tul-0 *FLC* homozygotes formed a distinct early-flowering class, verifying that the transgressive early-flowering phenotype is linked to Tul-0 *FLC*. In addition, Tul-0/Col *FLC* heterozygotes flowered later than the Tul-0 *FLC* homozygotes and earlier than the Col *FLC* homozygotes, indicating that the causative locus, like *FLC*, acts in a dosage-dependent manner (Lee et al. 1994).

Tul-0 *FLC* contains a transposable element in the first intron

PCR amplification of Tul-0 *FLC* revealed the presence of a large insertion, which, using additional primers, we delimited to a region at the beginning of the first intron. Sequencing this region revealed an 1181 bp transposable element, which was identical, both in its sequence and position, to sequence previously derived from the *FLC* allele of Kin-0, an early-flowering accession that, like Tul-0, was first collected in Michigan, USA (Lempe et al. 2005; Karlsson et al. 1993). Previous work has shown that the Kin-0 accession fails to complement an *flc* mutant, indicating that the Kin-0 *FLC* allele is weak or null and may contribute to Kin-0's early-flowering phenotype (Lempe et al. 2005). The Kin-0/Tul-0 *FLC* transposable element is also closely-related (>95% identical) to a 1224 bp transposable element at the end of the first intron of L*er FLC*, the presence of which has been shown to render L*er FLC* weak and to contribute to the early-flowering phenotype of the L*er* accession (Gazzani et al. 2003; Michaels et al. 2003; Liu et al. 2004). The relative positions of the Tul-0 *FLC* and L*er FLC* transposons are diagrammed in Figure 2b.

The close relationship and the physical proximity of the Tul-0 and L*er FLC* insertions suggested that, through transposition, one of these *FLC* alleles might have given rise to the other. Indeed, it is well-documented that some transposable elements, after excising from one genomic location, are more likely to reinsert at a nearby location than at a relatively distant location, a phenomenon that has been referred to as "local hopping" (Keller et al. 1993; Bancroft and Dean 1993; Tower et al. 1993; Zhang and Spradling 1993; Guimond et al. 2003). In addition, when a transposon inserts into a new genomic location, it often duplicates a short sequence of host DNA at the insertion location (Feschotte and Pritham 2007). When, subsequently, a transposon excises from this location, it often leaves behind it evidence of its previous residence, in the form

of a "footprint," which often consists of the duplicated host sequence or some mutagenized derivative thereof (Grappin et al. 1996; Scott et al. 1996; Feschotte and Pritham 2007).

Both the Tul-0 *FLC* and L*er FLC* insertions are flanked by 9 bp direct repeats, which, with reference to the Col *FLC* sequence, are duplications of the insertion site. However, examination of the site of the L*er FLC* insertion in Tul-0 and examination of the site of the Tul-0 *FLC* insertion in L*er* did not reveal any differences with reference to Col *FLC* sequence, indicating that, if either the Tul-0 *FLC* allele or the L*er FLC* allele had given rise to the other, element excision had left no footprint. The evidence therefore does not favor the theory that Tul-0 *FLC* and L*er FLC* are related by direct descent but instead supports two independent insertion events.

Although there did not seem to be a direct vertical link between the Tul-0 and L*er FLC* insertions, it still seemed possible that their proximity might still be explained by local hopping, i.e., perhaps these two independent insertion events had originated from a transposon reservoir close to *FLC*. To examine this model, we searched the Col genome for related transposons. This BLAST search revealed that, although there are no elements that are identical to either the Tul-0 or L*er FLC* insertions, there are three close relatives. However, none of these relatives is physically close to *FLC*, disfavoring a local hopping model.

Additional sequence analysis of the Tul-0 and L*er FLC* insertions, along with their three Col relatives, revealed that none of these transposons contain a predicted ORF large enough to encode a functional transposase. For this reason, these elements are likely nonautonomous, their transposition dependent upon a functional transposase provided in trans by an autonomous element (Feschotte and Pritham 2007). The source of the functional transposase therefore remains unclear. The first of these relatives is 1195 bp long and located at approximately 2.7 Mb

on Chromosome 4, on BAC clone C17L7; the second is 1242 bp long and located at approximately 16.8 Mb on Chromosome 5, on BAC clone MBK23; and the third is 1187 bp long and located at approximately 7.9 Mb on Chromosome 2, on BAC clone F24H14. Each of these elements, like the Tul-0 *FLC* and L*er FLC* insertions, is flanked by 9 bp direct repeat, confirming that members of this clade, like many transposons, insert into the host genome via a mechanism that involves the duplication of host DNA (Feschotte and Pritham 2007). Examination of these 9 bp direct repeat sequences (here, presented with reference to the 5' end of the element, defined as the end of Tul-0 element closest to the 5' end of *FLC*) reveals that this clade of transposable elements has a preference for AT-rich insertion sites: TTTCATTAT (Tul-0 *FLC*), ATTTAATAA (L*er FLC*), TTGAATATA (Col C17L7), AAAGTTATA (Col MBK23), and TTTTTTTAA (Col F24H14). Finally, each of these 5 related transposons, like many DNA transposons, contains, at its two termini, inverted repeats, which are likely necessary for transposition (Feschotte and Pritham 2007). These inverted repeats are not perfectly complementary; for instance, the inverted repeats of the Tul-0 *FLC* insertion are complementary only at 18 of the first 23 bp and at 24 for the first 40 bp.

At the same time, we also sequenced the remainder of the genomic Tul-0 *FLC*, from 300 bp before the start codon to 300 bp after the stop codon and found several differences with reference to Col *FLC* in addition to the presence of the transposon. These differences included, in the first intron, 4 single bp changes and a 30 bp deletion, and, within 300 bp after the stop codon, 3 single bp changes. Additional work in our laboratory has shown that many naturally occurring *FLC* alleles, both strong and weak, possess the same 30 bp intron deletion with relation to Col (data not shown). It has also been shown that a Col *FLC* construct engineered to

contain this 30 bp deletion creates a late-flowering phenotype when introduced into *flc*-null plants, indicating that this deletion does not compromise *FLC* strength (Michaels et al. 2003).

Tul-0 belongs to a clade of North American accessions

By quantitative PCR, we determined that the expression of Tul-0 *FLC* in its native Tul-0 background is lower than the expression of Col *FLC* in a FRI-Col background (Figure 2c). Late flowering despite low *FLC* expression has been previously described in a group of North America accessions, to which RRS-10, Knox-10, and Yo-0 belong, but from which Kin-0 appears to be phylogenetically distinct (Shindo et al. 2005; Nordborg et al. 2005). In that same study, these late-flowering North American accessions also exhibited a reduced sensitivity to vernalization (Shindo et al. 2005). The phenotypic similarities between Tul-0 and these accessions, together with the fact that each was originally collected in North America, suggested a phylogenetic relationship. Consistent with this conjecture, a previous study had concluded Tul-0 is closely related to Yo-0 (Schmid et al. 2006). To evaluate this relationship, we sequenced Tul-0 and several of these previously characterized accessions at over 100 pre-defined polymorphic markers (Clark et al. 2007) and found no differences between Tul-0 and RRS-10, suggesting that Tul-0 is closely related to these North American accessions and may in fact be identical to RRS-10. We also found that these North American accessions possessed the same transposon-containing *FLC* allele as Tul-0, further underscoring this phylogenetic relationship.

These facts together suggested that Tul-0 and the related RRS-10, Knox-10, and Yo-0, all of which are late-flowering, might possess additional loci that compensated for their weak, poorly-expressed *FLC* allele. In contrast, the more distantly-related Kin-0, which possesses the same weak *FLC* allele but is early-flowering, might lack such compensatory loci.

We subjected several of these Tul-0 relatives, together with FRI-Col and Tul-0, to a vernalization time course consisting of 0, 40, 80, and 120 day time points (Figure 3). Yo-0, Rmx-A02, Pna-10, Knox-10, and RRS-10 behaved much like Tul-0, remaining late-flowering (50-80 leaves) following 40 days of vernalization but becoming early-flowering following subsequent months of cold treatment. Knox-18, and Pna-17, however, behaved more like FRI-Col, flowering with approximately 20 leaves after 40 days of vernalization, indicating that the kinetics of the vernalization response differ among members of this clade.

Tul-0 *FLC* and L*er* *FLC* confer similar flowering phenotypes in L*er* and FRI-Col backgrounds

Because L*er* *FLC* and Tul-0 *FLC* are so similar molecularly, we hypothesized that, when placed in the same background, they might also confer similar flowering phenotypes. To test this idea, we selected a stable, early-flowering backcross 9 line containing Tul-0 *FLC* in a FRI-Col background, which we called Tul-0 *FLC* FRI-Col. In parallel, we also introgressed L*er* *FLC* into a FRI-Col background to create an early-flowering backcross 5 line containing L*er* *FLC* in a FRI-Col background, which we called L*er* *FLC* FRI-Col. In addition, we also introgressed Tul-0 *FLC* into a L*er* background (backcross 5), a line we called Tul-0 *FLC* L*er*. For each of four lines: L*er* *FLC* in a L*er* background (L*er*), Tul-0 *FLC* in a L*er* background (Tul-0 *FLC* L*er*), L*er* *FLC* FRI-Col, and Tul-0 *FLC* FRI-Col, we determined the total leaf number at flowering (Figure 4a). The two lines in the L*er* background flowered at approximately the same time, as did the two lines in a FRI-Col background, suggesting a similar impairment in L*er* and Tul-0 *FLC* function.

In order to compare the strength of L*er* and Tul-0 *FLC* in a segregating population descended from a single parent, we crossed two NILs, Ler *FLC* FRI-Col and Tul-0 *FLC*, to

create a Ler/Tul-0 *FLC* heterozygote in a FRI-Col background. We planted more than 60 offspring of this Ler/Tul-0 *FLC* heterozygote and for each plant determined both the total leaf number at flowering and the genotype at *FLC* (Figure 4b). In this population, L*er FLC* offspring flowered later (approximately 5 leaves) than Tul-0 *FLC* offspring, indicating that Ler *FLC* may be a stronger allele than Tul-0 *FLC*. Why the greater strength of L*er FLC* should be apparent in this segregating population but not in the comparison of the two fixed lines is not clear, though it perhaps points to the persistence of modifier loci in the two fixed lines.

Tul-0 *FLC* is functional

The fact that Tul-0 *FLC* appeared to be weaker even than L*er* FLC, which is quite weak, raised the possibility that Tul-0 *FLC* might be entirely nonfunctional. Since the effect of both *FRI* and the autonomous pathway on flowering time has been previously shown to depend entirely upon the presence of a functional *FLC* allele (Michaels and Amasino 2001), we decided to address this question by examining the response of Tul-0 *FLC* lines to the loss of *FRI* and the autonomous pathway. If Tul-0 *FLC* were functional, the flowering time of Tul-0 *FLC* lines would be accelerated by *fri* mutations but delayed by autonomous pathway mutations. If, conversely, Tul-0 *FLC* were not functional, flowering time would be unaffected by the introduction of these mutations. L*er FLC*, which confers a flowering time delay similar to that conferred by Tul-0 *FLC* and whose functionality has been previously demonstrated, would serve as a functional *FLC* control (Koornneef et al. 1991).

To conduct this experiment, we crossed both Tul-0 *FLC* FRI-Col and L*er FLC* FRI-Col to Col, which harbors a nonfunctional *fri* allele, and to FRI-Col plants harboring null mutations in the autonomous pathway genes *FCA*, *FLK*, *FPA*, *FLD*, *FVE*, and *LD*. In the next generation, we selected 14 lines containing two copies of either Tul-0 *FLC* or L*er FLC*, and two copies of a

null allele of one of the 7 additional flowering-time regulators. We planted these 14 lines, together with the parental Tul-0 *FLC* FRI-Col and L*er FLC* FRI-Col lines, and determined the average number of leaves formed at flowering (Figure 5a).

Both the *fri* Tul-0 *FLC* and *fri* L*er FLC* lines flowered earlier than the corresponding *FRI* parents, indicating that Tul-0 *FLC*, like L*er FLC*, is responsive to *FRI*. Consistent with this flowering behavior, we also found that the expression of Tul-0 *FLC* in the parental *FRI* line was higher than *FLC* expression in the corresponding *fri* line (Figure 5b), indicating that, as with the strong Col *FLC*, *FRI* increases the level of Tul-0 *FLC* mRNA (Michaels and Amasino 1999).

In the L*er FLC* lines, introduction of all six autonomous pathway mutations delayed flowering with respect to the L*er FLC* FRI-Col parent (Figure 5a). With the exception of *flk*, which has not, to our knowledge, been previously coupled to L*er FLC*, this was consistent with previous reports (Koornneef et al. 1991, 1994, and 1998; Lee et al. 1994; Sanda and Amasino 1996). In contrast, in the Tul-0 *FLC* lines, the introduction of *fca*, *flk*, and *fve* mutations delayed flowering with respect to the *FLC* FRI-Col control, while, unexpectedly, the introduction of *fpa*, *fld*, and *ld* mutations accelerated flowering (Figure 5a). In these Tul-0 *FLC* lines, *FLC* expression mirrored flowering behavior: *FLC* expression was elevated in *fca*, *flk*, and *fve* mutants but reduced in *fpa*, *fld*, and *ld* mutants (Figure 5c).

The unexpected behavior of the *fpa*, *fld* and *ld* mutant Tul-0 *FLC* lines raised the possibility that, during their construction, these lines had somehow inherited an additional locus that accelerated flowering by a means unrelated to the autonomous pathway mutation. In order to address this concern, we reconstructed five (*fpa* omitted) of the Tul-0 *FLC* autonomous mutant lines again independently, employing both an independently-produced Tul-0 *FLC* (#2) FRI-Col backcross 5 line and a set of autonomous pathway mutants in a *fri* background rather

than, as previously, in a *FRI* background. The relative flowering behavior of these new *fri* lines was similar to those in Figure 5a, confirming both the flowering-time delay caused by the *fca*, *flk*, and *fve* mutations and the failure of the *fld* and *ld* mutations to exert a similar delay (Figure 5d). Interestingly, however, in contrast to the lines in Figure 5d, the new *fld* and *ld* mutant Tul-0 *FLC* lines flowered no earlier than the control; whether this difference is due to the absence of FRI function or to differences in other genomic regions remains unclear (Figure 5d).

Together, the fact that both the flowering time and the *FLC* expression of the Tul-0 *FLC* FRI-Col line are elevated by the introduction of functional FRI and of *fca*, *flk*, and *fve* mutations indicates that, like L*er FLC*, Tul-0 *FLC* is functional. The behavior of the Tul-0 *FLC fpa*, *fld*, and *ld* mutants remains unexplained, although it might be speculated to be due to cis effects resulting from differences in the sequence of L*er* and Tul-0 *FLC*, including, for instance, the different intronic positions of their respective transposable elements (see Discussion).

DISCUSSION

In this work, we examined the flowering behavior of Tul-0, an *Arabidopsis* accession from Michigan, USA, both before and after vernalizing cold treatment. We confirmed earlier observations that Tul-0 is late-flowering before and after one month of vernalization (Karlsson et al. 1993), but, in addition, we showed that flowering is substantially accelerated by additional months of cold treatment. In the course of mapping loci responsible for this reduced vernalization sensitivity, we revealed a still more striking phenotype: in Tul-0-derived mapping populations, some plants flower early even in the absence of vernalization. We mapped this phenotype to the Tul-0 *FLC* allele, which is poorly-expressed with reference to Col *FLC*, and, like many naturally-occurring weak *FLC* alleles, contains a transposable element in the first

intron. We compared Tul-0 *FLC* to Ler *FLC*, a naturally occurring allele that contains a closely related transposon, and showed that these two alleles confer similar although not identical flowering phenotypes. We also demonstrated that, although weak, Tul-0 *FLC* is functional, and that it responds to *FRI* and to some—but, curiously, not to all—members of the autonomous pathway.

The fact that Tul-0 *FLC* lines exhibit atypical responses to the introduction of *fpa*, *fld*, and *ld* mutations, whereas *Ler FLC* lines exhibit typical responses to the introduction of the same mutations, must be due to some fundamental difference between the Tul-0 *FLC* and *Ler FLC* lines. The most obvious difference between these lines is at *FLC*, and, at *FLC*, the most prominent difference is the relative position of the transposons within the first intron, with the Tul-0 element residing at the beginning of the intron and the L*er* element residing at the end of the intron. It is possible that cis elements required for the response of *FLC* to some members of the autonomous pathway may be present at the beginning of the intron, in a region disrupted by the Tul-0 transposon. Indeed, a deletion analysis of *FLC* has previously shown that removal of the 313-607 region in *FLC* (as measured from the start codon) phenocopies the loss of *FLD*, suggesting a model in which *FLD* or its downstream effectors interfaces with the *FLC* through an element within this approximately 300 bp region (He et al. 2003). Interestingly, the Tul-0 *FLC* transposon is located within this region, at position 491. It is therefore possible that the Tul-0 transposon disrupts a critical cis element, rendering Tul-0 *FLC* immune from *FLD*-mediated repression. Still, this model fails to explain why, even given these circumstances, the loss of *FLD* function should accelerate flowering with reference to the *FLD*+ control. In another model, Tul-0-specific *FLC* sequence might fundamentally alter the nature of the interaction between *FLC* and *FPA*, *FLD*, and *LD* or their downstream effectors, causing the latter to recruit

transcription activators rather than repressors to *FLC*. The loss of *FPA*, *FLD*, and *LD* function would therefore reduce *FLC* expression and accelerate flowering.

Additional models, involving differences in non-*FLC* genes that may not have been removed during *FLC* introgression, might also be proposed. A Tul-0 locus might, in trans, specifically prevent the function of FPA, FLD, and LD, but not the function of the other members of the autonomous pathway, such that the loss of *FPA*, *FLD*, and *LD* would produce no additional phenotype. Again, however, why the loss of autonomous pathway function would cause these lines to flower earlier than the control remains unclear.

Members of the autonomous pathway have been implicated in the regulation of the expression of many non-*FLC* genes, including in the repression of some transposable elements (Wilson et al. 2005; Marquardt et al. 2006; Bäurle et al. 2007; Bäurle and Dean 2008; Veley and Michaels 2008). If some members of the autonomous pathway reduce Tul-0 *FLC* expression through the repression of transposable element, the sudden removal of this repression through loss of autonomous pathway function might, paradoxically, render *FLC* subject to more intense silencing through other pathways and reduce its expression. In a transposon-silencing model, however, it is unclear why the autonomous pathway should exert different effects on the closely related Tul-0 *FLC* and L*er FLC* transposons, unless perhaps the fact that the Tul-0 transposon is located closer to the *FLC* promoter affects the manner in which it is silenced.

Certain members of the autonomous pathway might also moderate the expression of certain non-*FLC* Tul-0 loci that persist in the Tul-0 *FLC* lines following introgression. If one of these loci in turn moderates flowering time, then the loss of autonomous pathway function might alter the previous balance in favor of floral accelerators and, in sum, accelerate flowering time.

The fact that *fpa*, *fld*, and *ld* mutations have different effects upon Tul-0 *FLC* lines than *fca*, *flk*, and *fve* mutations is perhaps still more curious when one considers that FPA, FLD, and LD belong to three different mechanistic subdivisions of the autonomous pathway. FPA has been proposed to act through RNA processing, FLD through chromatin modification, and LD, which contains a homeodomain, may interact with DNA (Simpson 2004). However, a recent study may support the idea that FPA, FLD, and LD, may in some respects function together. The acceleration of flowering caused by the overexpression of FPA is suppressed by the loss of FLD, suggesting a model in which, if FLD cannot function, as, for instance, because of the loss of a required cis element in Tul-0 *FLC*, FPA may not function either (Bäurle and Dean 2008). However, FCA also requires FLD function, and yet, in contrast to the *fld* allele, the *fca* allele delays the flowering of Tul-0 lines (Liu et al. 2007). Finally, an analysis of gene expression in autonomous pathway mutants has suggested that FPA and LD may function redundantly to regulate many of the same genes as FLD (Veley and Michaels 2008). If the atypical responses of the Tul-0 *FLC* lines to the introduction of *fpa*, *fld*, and *ld* mutations is due to changes in the expression of some non-*FLC* gene, it may therefore not be surprising that the loss of these three genes might exert the same effect.

The fact that Tul-0 *FLC* contains a transposon in the first intron compounds the already curious circumstance that multiple natural *FLC* alleles contain such transposons, apparently independent events. Further compounding this seeming unlikelihood is the fact that Tul-0 *FLC* and L*er FLC* contain highly related transposable elements, although on opposite sides of the first intron and in reverse orientation to one another. The low probability of this circumstance is further underscored by the fact that, at least in the Col background, this particular clade of transposable elements exists in low copy number, with only three close relatives on

Chromosomes 4, 5, and 2, respectively. Of course, it is possible that this element may be much more common in accessions other then Col, which might render its independent insertion into both Tul-0 *FLC* and L*er FLC* less exceptional.

Although the existence of multiple *FLC* insertions does seem curious, a strikingly similar example is nonetheless found in the medaka fish *Oryzias latipes*. In natural populations of *Oryzias latipes*, there are at least three different weak or nonfunctional alleles of the melanin biosynthesis gene *TYROSINASE*, the attenuation of which reduces pigmentation and the loss of which creates albinism (Oetting et al. 2003). These three natural alleles arose, respectively, through the insertion of a 4.7 kb Tol2 element in the 5' UTR, the insertion of a 1.9 kb Tol1 element in the first exon and the insertion of a 4.7 kb Tol2 element in the fifth exon (Iida et al. 2004; Koga et al. 1995 and 1996). The occurrence of three different transposon insertions in the same gene, two of which are Tol2 elements, is still more striking when one considers that Tol2 is a low copy element, with only about 20 copies in the diploid *Oryzias latipes* genome (Koga and Hori 2000; Koga et al. 2006).

A basic similarity between the functions of *FLC* and *TYROSINASE* suggest two reasons why the rate at which different transposon insertions are identified as contributing to natural variation might be higher within these two genes than within other genes in their respective genetic backgrounds. Attenuation or loss of function of *FLC* in *A. thaliana* and of *TYROSINASE* in *O. latipes* lead to dramatic—but not deleterious—phenotypic changes, reducing flowering time in the former and coloration in the latter. These changes have immediate adaptive consequences, which may be advantageous in some environments, with the effect that transposon-containing alleles, which may arise at only a low frequency, may be favored by selection and may be more likely to persist. Thus, transposon-containing alleles of *FLC* and

TYROSINASE may be identified disproportionately frequently because they persist in nature to a greater degree. A second reason that transposon-containing alleles of *FLC* and *TYROSINASE* might be identified disproportionately frequently is that, because a change in the function of either gene creates such an easily scorable phenotypic change, such natural alleles are readily mapped. In contrast, the insertion of transposons into genes whose attenuation produces only a slight or ambiguous phenotype, or perhaps a phenotype which specialized training is necessary to detect, will only rarely attract investigators.

In well-characterized accessions such as Col, a strong *FLC* allele is required to create a late-flowering phenotype. The fact that the weak Tul-0 *FLC* is able to confer a late-flowering phenotype in a Tul-0 background suggests the existence of compensatory loci, which act to delay flowering in a Tul-0 background. Such compensatory loci might act to potentiate the weak *FLC* or might instead delay flowering by an *FLC*-independent mechanism. The identification of such loci, which have not have not been previously characterized, may augment our understanding of the mechanisms that underlie floral timing. Further, the identification of such loci may make it possible, by comparing the sequence of the late-flowering Tul-0 to that of the early-flowering Kin-0, to retrace the evolution of the North American clade. Specifically, it will be interesting to determine (1) whether the ancestor of the late-flowering North American clade was early-flowering, like Kin-0, and only later acquired compensatory loci that rendered it late-flowering, or (2) whether instead this ancestor possessed both a strong *FLC* and these compensatory loci, producing an exceptionally late-flowering phenotype that was only later moderated by the acquisition of a weak *FLC* allele.

ACKNOWLEDGMENTS

This project was initiated by Sibum Sung, a former UW-Madison post-doc and now assistant professor at the University of Texas at Austin. Sibum Sung introgressed Tul-0 loci required for vernalization insensitivity into a FRI-Col background out to the backcross 2 generation. Additional work in this chapter was completed with the assistance of UW-Madison undergraduates Jennifer Kraninger, Libby Karn, and Curren Sharma.

Figure 1. Vernalization time course. Total leaf number at flowering of FRI-Col (left) and Tul-0 (right) following 0, 1, 2, 3, 4, 5, and 6 months of cold treatment (4°C). Each data bar represents 18 plants. Here, a month is defined as 30 days.

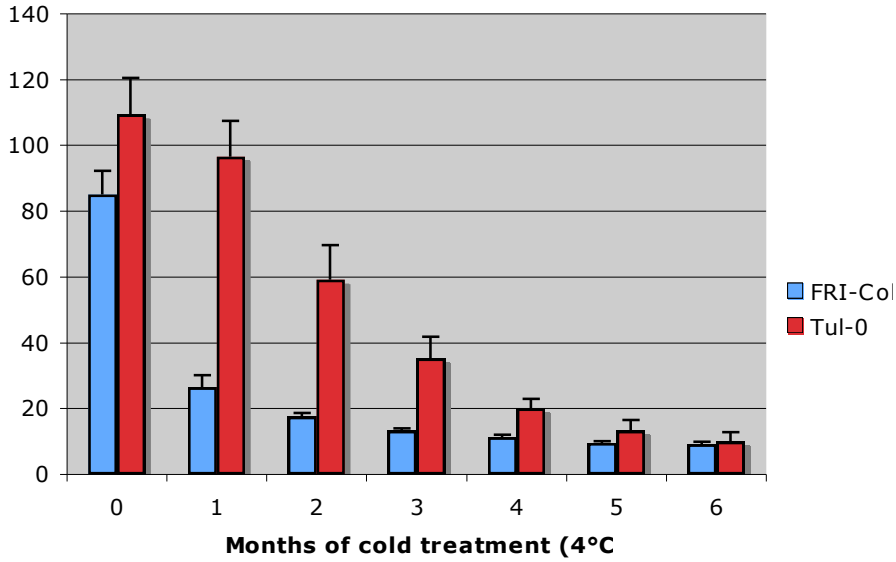
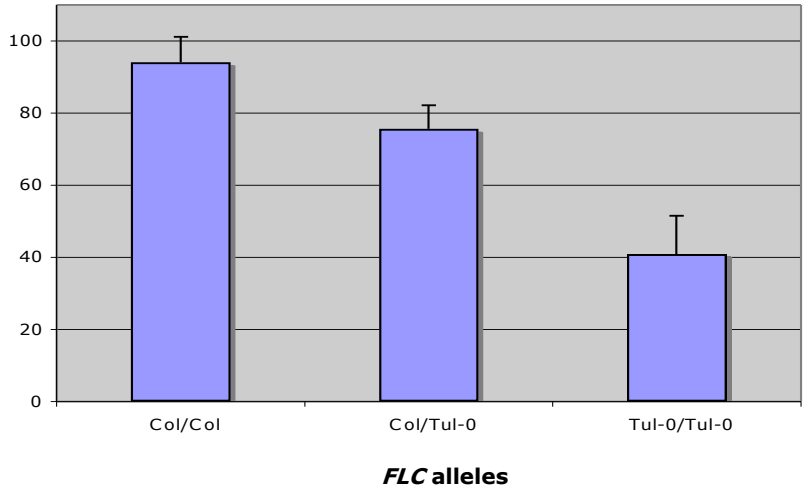
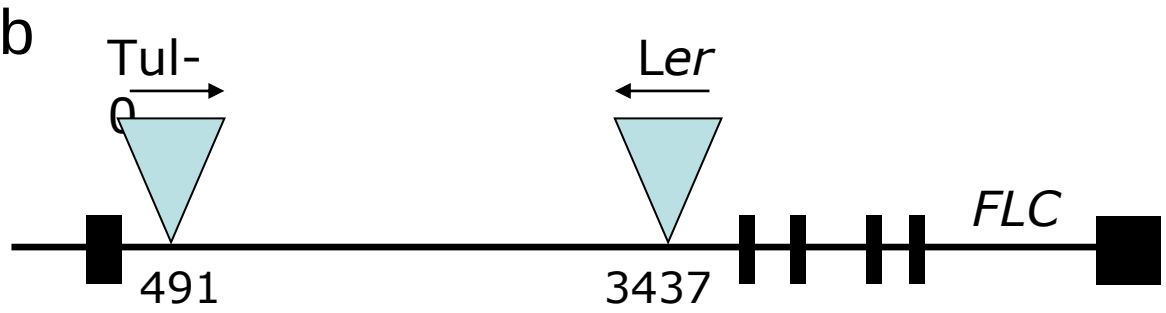

140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Months of cold treatment (4°C
FRI-Col
Tul-0

Figure 2. Phenotypic and molecular characterization of Tul-0 *FLC* (no vernalization). (a) In a FRI-Col background, an accelerated flowering phenotype segregates with Tul-0 *FLC*. Total leaf number at flowering of Col *FLC* (left), heterozygous *FLC* (middle) and Tul-0 *FLC* (right) offspring of a backcross 10 F1 (FRI-Col background). Population was divided as follows: 14 Col *FLC*, 24 heterozygous, and 13 Tul-0 *FLC* plants. (b) Tul-0 *FLC* contains a transposon at the beginning of the first intron; L*er FLC* contains a highly similar transposon at the end of the first intron. Black rectangles represent *FLC* exons and inverted triangles represent transposons. The arrows indicate that the Tul-0 and L*er* transposons are oppositely oriented with reference to one another, with the Tul-0 transposon arbitrarily used to define the forwards direction. Numbers indicate the bp position of each insertion with reference to the *FLC* start codon. (c) Relative *FLC* expression in FRI-Col (left) and Tul-0 (right).

a


100
80
60
40
20
0
Col/Col
Col/Tul-0
Tul-0/Tul-0
FLC alleles

b


Tul-0
Ler
FLC
491
3437

c

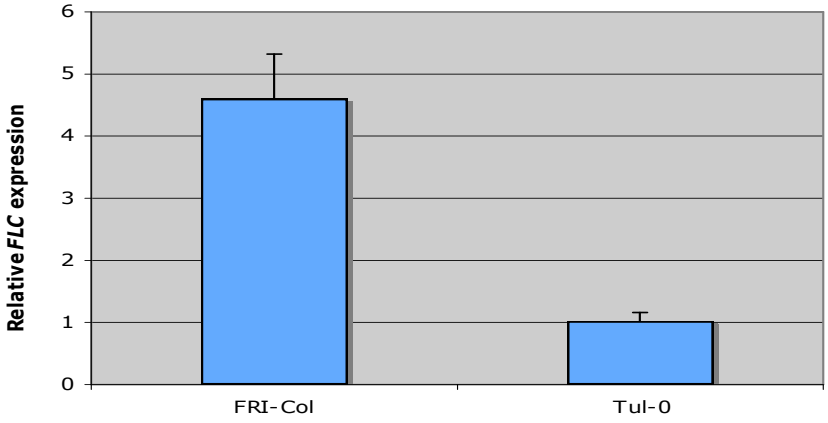

6
5
4
3
2
1
0
Relative FLC expression
FRI-Col
Tul-0

Figure 3. Vernalization time course. Total leaf number at flowering of FRI-Col, Tul-0 and several additional North American accessions following 0, 40, 80, and 120 days of vernalization. Each data bar represents at least 16 plants. Plants that had not flowered at experiment termination were assigned a value of 120 leaves.

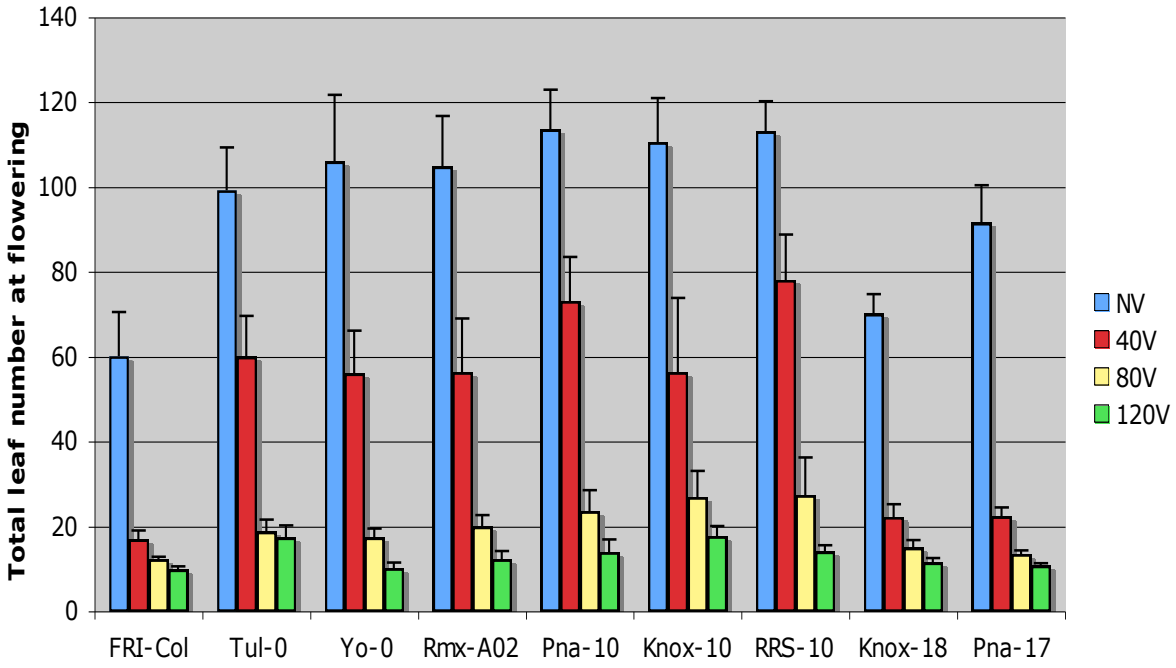
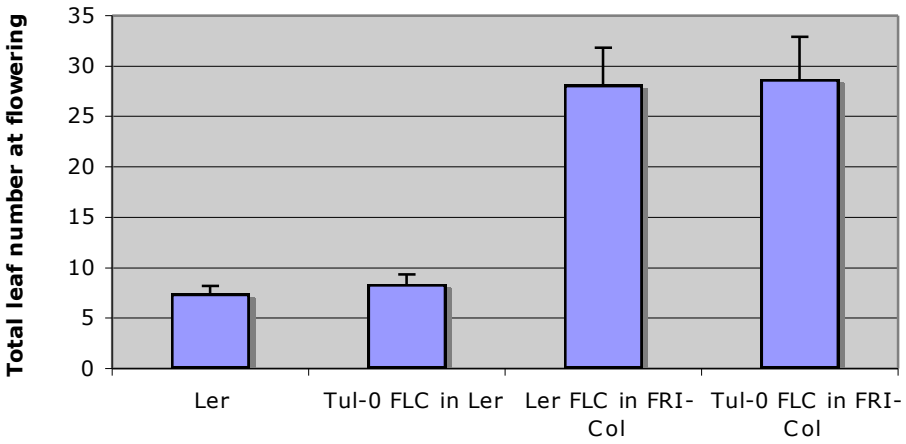

Total leaf number at flowering
140
120
100
80
60
40
20
0
FRI-Col
Tul-0
Yo-0
Rmx-A02
Pna-10
Knox-10
RRS-10
Knox-18
Pna-17
NV
40V
80V
120V

Figure 4. L*er FLC* and Tul-0 *FLC* confer similar flowering phenotypes. (a) On left, L*er* background: total leaf number at flowering of L*er* and of Tul-0 *FLC* L*er*. On right, FRI-Col background: total leaf number at flowering of L*er FLC* FRI-Col and Tul-0 *FLC* FRI-Col. Each data bar represents at least 15 plants. (b) Total leaf number at flowering of L*er FLC* (left), heterozygous *FLC* (middle) and Tul-0 *FLC* (right) offspring of a L*er FLC*/Tul-0 *FLC* F1 (FRI-Col background). Offspring were divided as follows: L*er FLC,* 25 plants; heterozygous *FLC*, 28 plants; and Tul-0 *FLC*, 14 plants. The difference between the L*er FLC* and Tul-0 *FLC* classes (starred bars) is statistically significant ($p<.005$, Student's t-test).

a


Total leaf number at flowering
35
30
25
20
15
10
5
0
Ler
Tul-0 FLC in Ler
Ler FLC in FRI-Col
Tul-0 FLC in FRI-Col

b

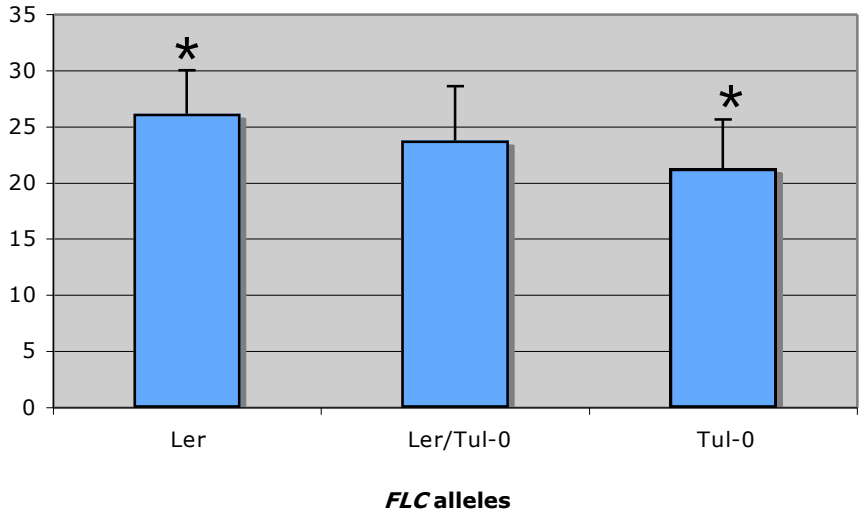
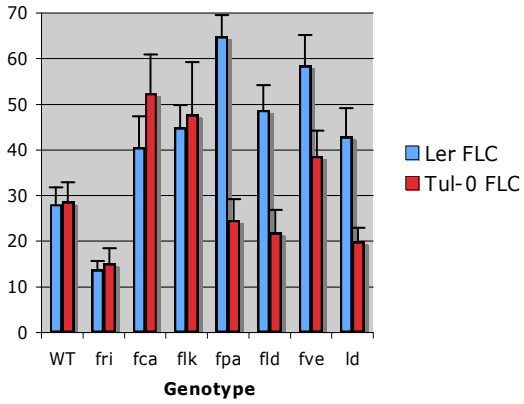
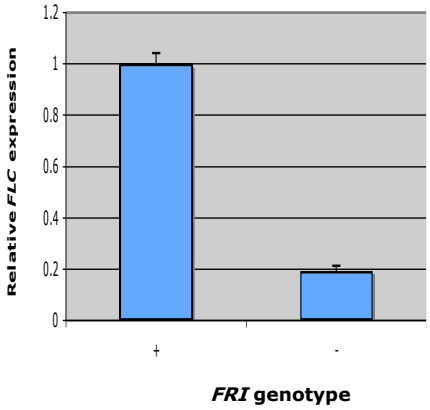

35
30
25
20
15
10
5
0
*
*
Ler
Ler/Tul-0
Tul-0
FLC alleles

Figure 5. Response of Tul-0 *FLC* lines to the introduction of *fri* and autonomous pathway mutations (FRI-Col background). (a) Total Leaf number at flowering of lines with L*er FLC* (left) and Tul-0 *FLC* (right) in *fri* and in *FRI* autonomous pathway mutant backgrounds. "WT" refers to L*er FLC* FRI-Col and Tul-0 *FLC* FRI-Col, i.e., to control lines without *fri* or autonomous pathway mutations. Each data bar represents at least 17 plants. (b) Relative expression of Tul-0 *FLC* in lines with functional *FRI* (+, left) and nonfunctional *fri* (-, right). (c) Relative expression of Tul-0 *FLC* in a control line ("WT") and in *fca*, *flk*, *fpa*, *fld*, *fve*, and *ld* mutant lines (FRI-Col background). (d) Total leaf number at flowering of an independently produced set of lines (#2) with Tul-0 *FLC* in *fri* and *fri fca*, *fri flk*, *fri fld*, *fri fve*, and *fri ld* mutant backgrounds. "WT" refers to *fri* Tul-0 *FLC* (#2) Col, i.e., to a control line without autonomous pathway mutations (Col background). Each data bar represents at least 8 plants.

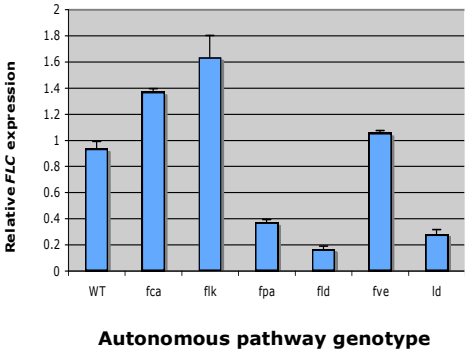
a


70
60
50
40
30
20
10
0
WT
fri
fca
flk
fpa
fld
fve
ld
Genotype
Ler FLC
Tul-0 FLC

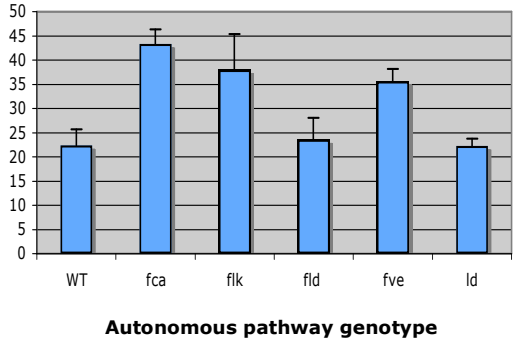
b


1.2
1
0.8
0.6
0.4
0.2
0
Relative FLC expression
+
-
FRI genotype

c


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative FLC expression
WT
fca
flk
fpa
fld
fve
ld
Autonomous pathway genotype

d


50
45
40
35
30
25
20
15
10
5
0
WT
fca
flk
fld
fve
ld
Autonomous pathway genotype

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CHAPTER 3

MULTIPLE TUL-0 LOCI CREATE A LATE-FLOWERING PHENOTYPE IN THE PRESENCE OF A WEAK *FLC* ALLELE

ABSTRACT

Natural accessions of *Arabidopsis thaliana* exhibit substantial variation in the timing of floral initiation, and much of this variation is due to allelic differences at *FRI* (*FRIGIDA*) and *FLOWERING LOCUS C* (*FLC*). In most backgrounds, strong alleles of both *FRI* and *FLC* are required to create a late-flowering phenotype and mutational attenuation of either gene results in an early-flowering phenotype. In the following work, we examine the unusual case of Tul-0, a North American accession, which, despite possessing a weak *FLC* allele, is nonetheless late-flowering. Using near-isogenic lines (NILs), created both by the introgression of Tul-0 loci into laboratory accessions and by the introgression of loci from laboratory accessions into Tul-0, we begin to dissect the genetic basis of this phenotype and define a role for at least two Tul-0 loci. The first, *LATE INDEPENDENT of FLC* (*LIF*), is located at the top of Chromosome V and delays flowering via an *FLC*-independent mechanism. The second, *FLC-ENHANCER 1* (*FEN1*), is located at the bottom of Chromosome I and delays flowering via an *FLC*-dependent mechanism. In addition, we show that Tul-0 enhancer loci do not act exclusively on the weak Tul-0 *FLC* allele but also enhance the weak L*er* and Da (1)-12 *FLC* alleles, as well as the strong Col *FLC* allele. Finally, we show that the late-flowering phenotype is in part *FRI*-independent. Our work illustrates the utility of employing advanced NILs to dissect novel flowering phenotypes and points to the existence of new *FLC*-enhancer loci that, in addition to *FRI*, contribute to flowering-time variation.

INTRODUCTION

Different environments favor different developmental strategies. These pressures influence evolution, contributing, on a grand scale, to speciation, and, on a smaller scale, to

variation among geographically separate populations belonging to the same species. Natural accessions of the model plant *Arabidopsis thaliana,* more than 750 of which have been collected from sites on four continents, present an opportunity to study differences in developmental timing within a species and to document evolutionary processes as they occur on a small scale.

Among these *A. thaliana* accessions, variation in the timing of flower production has been particularly well-documented, and, under laboratory conditions, these differences are indeed striking. When quantified in terms of the number of vegetative leaves formed prior to the production of the first flower, this variation extends across a 10-fold scale, with the earliest *A. thaliana* accessions flowering with fewer than 10 vegetative leaves and the latest accessions flowering with more than 100 leaves (Shindo et al. 2005; Werner et al. 2005a; Lempe et al. 2005). It has been hypothesized that this variation reflects the adaptation of natural accessions to different environments, and, in support of this idea, studies using European accessions do show correlations between flowering time and latitude of origin, although these relationships are complex (Johanson et al. 2000; Stinchcombe et al. 2004 and 2005; Shindo et al. 2005; Lempe et al. 2005).

On a molecular level, much of this variation is due to allelic differences at two genes, *FLOWERING LOCUS C* (*FLC*) and *FRI* (*FRIGIDA*), which encode floral repressors that act in the same pathway. FLC is a MADS box transcription factor, functional forms of which delay flowering by repressing the expression of floral integrators (Sheldon et al. 1999; Michaels and Amasino 1999; Michaels et al. 2005; Hepworth et al. 2002; Searle et al. 2006). FRI is a plant-specific coiled-coil domain protein, functional forms of which delay flowering by upregulating *FLC* (Johanson et al. 2000; Michaels and Amasino 1999). In most accessions, strong alleles of both *FLC* and *FRI* are required to create a late-flowering phenotype. As the ancestral *A. thaliana*

diversified into distinct accessions, there have been several independent inactivating or attenuating mutations in *FLC* or *FRI* that have created early-flowering accessions from late-flowering progenitors (Gazzani et al. 2003; Michaels et al. 2003; Lempe et al. 2005; Werner et al. 2005a; Johanson et al. 2000; Le Corre et al. 2002; Shindo et al. 2005).

Many proteins are involved in determining *FLC* expression levels, both endogenously and in response to external temperature cues. Members of the autonomous pathway constitutively downregulate *FLC* expression; conversely, many other proteins, including FRI, constitutively upregulate *FLC* expression (Simpson 2004; Kim et al. 2009). Still another set of proteins downregulate *FLC* expression contingent upon the plant's experience of prolonged winter cold, during a process called vernalization (Sung and Amasino 2005; De Lucia et al. 2008). In nature, vernalization, combined with the photoperiod response, serves to accelerate flowering following the return of spring (Simpson and Dean 2002). The photoperiod response, in contrast to vernalization, operates via *FLC*-independent mechanisms (Michaels 2008).

Current understanding of flowering-time regulation is based in large part upon work with a small number of laboratory lines, including Landsberg *erecta* (L*er*), Columbia (Col), and the Col-derived FRI-Col. However, many *A. thaliana* accessions exhibit alternate flowering behaviors, the molecular explanations for which remain unknown. Some accessions are early-flowering despite relatively high *FLC* expression, others are late-flowering despite null alleles of *FLC* or *FRI* or weak *FLC* expression, and others are insensitive to vernalizing cold treatment (Shindo et al. 2005 and 2006; Werner et al. 2005a; Lempe et al. 2005). These novel behaviors suggest that additional flowering-time regulators, which may act independently of *FLC* or may govern *FLC* action via an unknown mechanism, may contribute to flowering-time variation.

In an effort to define such regulators, we continue work with Tul-0, a vernalization-insensitive accession that is late-flowering despite possessing a weak *FLC* allele (chapter 2, this work). Using near-isogenic lines (NILs), we define two Tul-0 loci that compensate for the weak *FLC* and contribute to the late-flowering phenotype. The first, *LATE INDEPENDENT of FLC* (*LIF*), is linked to a 4.6Mb marker on Chromosome V and acts independently of *FLC*. The second, *FLC-Enhancer 1* (*FEN1*), is linked to a 21.2 Mb marker on Chromosome I and requires an active *FLC* allele to delay flowering. We also show that, in a Tul-0 background, *FLC* enhancement (1) is accompanied by an elevation of *FLC* expression, (2) affects not only the Tul-0 *FLC* allele but also the L*er*, Da (1)-12, and Col *FLC* alleles, and (3) is in part *FRI*-independent. Our results support a model in which multiple Tul-0 loci, acting independently and via distinct mechanisms, create a late-flowering phenotype in the presence of the weak Tul-0 *FLC*.

MATERIALS AND METHODS

Plant materials:

The accessions Tul-0 and Col, the laboratory NIL FRI-Col, and the alleles L*er FLC* and Col *fri* are as discussed in chapter 2.

FRI-Col *flc-3* has been previously described (Michaels and Amasino 1999).

The retrotransposon-containing Da (1)-12 *FLC* allele has been previously described (Michaels and Amasino 2003).

Bur-0 seeds were provided by Todd Michael and Julin Maloof. Bur-0 has been previously described (Werner et al. 2005a).

Line description:

Introgression lines were constructed as described in chapter 2.

For the purpose of line construction, *LIF* was defined as a region between 4.6 Mb and 6.8 Mb on Chromosome V and *FEN1* was defined as a region between 21.2 Mb and 24.6 Mb on Chromosome I.

The construction of Tul-0 *FLC* FRI-Col, L*er FLC* FRI-Col, *fri* Tul-0 *FLC* Col and *fri* L*er FLC* Col is described in chapter 2.

Tul-0 *flc-3* was created by introgressing the *flc-3* allele from FRI-Col *flc-3* into a Tul-0 background out to the backcross 7 generation. Tul-0 *flc-3* is Col at *LIF* (here, defined phenotypically).

Tul-0 *flc-3*+Tul-0 *LIF* was generated from the same population as Tul-0 *flc-3*, but, in contrast to Tul-0 *flc-3*, is descended from a recombinant specifically selected to include Tul-0 genomic material beneath 4.6 Mb on Chromosome V, a region physically linked to *FLC*.

FRI-Col+Tul-0 *FEN1* was created by introgressing the Tul-0 *FEN1* region into a FRI-Col background out the backcross 7 generation.

Tul-0 *FLC* FRI-Col+Tul-0 *FEN1* was created through genetic combination of Tul-0 *FLC* FRI-Col and FRI-Col+Tul-0 *FEN1*.

Tul-0 *FLC* FRI-Col+Tul-0 *LIF* was created by introgressing the top part of Tul-0 Chromosome V (*FLC* + *LIF*) into a FRI-Col background out to the backcross 9 generation.

Tul-0+Col *FEN1* was created by introgressing the *FEN1* region from FRI-Col into a Tul-0 background out to the backcross 6 generation.

Tul-0+Col *LIF* was created by introgressing the top part of Col Chromosome V into a Tul-0 background out to the backcross 6 generation. At the final generation, a recombinant was

selected specifically to retain Tul-0 *FLC* but to exclude the Tul-0 *LIF* region; thus, this line is Tul-0 at *FLC* but Col at the *LIF* region (Tul-0 background).

Da *FLC* FRI-Col and Da *FLC* Tul-0 were created by introgressing Da (1)-12 *FLC* into FRI-Col and Tul-0, respectively, out to the backcross 5 generations. No additional selection was imposed, and both lines are Da (1)-12 at the *LIF* region.

L*er FLC* FRI-Col+Tul-0 *FEN1* was created through the genetic combination of L*er FLC* FRI-Col and FRI-Col+Tul-0 *FEN1* (both described above).

L*er FLC* Tul-0 was created through the introgression of L*er FLC* into a Tul-0 background out to the backcross 7 generation. At the final generation, a recombinant was selected specifically in order to exclude the L*er LIF* region; thus, L*er FLC* Tul-0 is Tul-0 at the *LIF* region.

Col *FLC* Tul-0 was created through the introgression of Col *FLC* into a Tul-0 background out to the backcross 6 generation. This line is Col at the *LIF* region.

Col *FLC* Tul-0+Tul-0 *LIF* was created through the introgression of Col *FLC* into a Tul-0 background out to the backcross 6 generation. In the final generation, a Chromosome V recombinant was selected; thus, this line is Tul-0 at the *LIF* region.

fri Tul-0 was created by introgressing the nonfunctional *fri* allele from Col into a Tul-0 background out to the backcross 8 generation.

fri Tul-0 *FLC* Col+Tul-0 *LIF* was created through the genetic combination of Col and Tul-0 *FLC* FRI-Col+Tul-0 *LIF* (described above), to create a backcross 10 line (Col background).

fri L*er FLC* Tul-0 was created through genetic combination of L*er FLC* Tul-0 and *fri* Tul-0 (described above) (Tul-0 background). This is line is Tul-0 at the *LIF* region.

fri Col+Tul-0 *FEN1* was created through genetic combination of FRI-Col+Tul-0 *FEN1* and (*fri*) Col (Col background).

fri Col *FLC* Tul-0+Tul-0 *LIF* was created through genetic combination of Col *FLC* Tul-0+Tul-0 *LIF* and *fri* Tul-0 (both described above) (Tul-0 background).

fri Tul-0 *flc-3*+Tul-0 *LIF* was created through the genetic combination of *fri* Tul-0 and Tul-0 *flc-3*+Tul-0 *LIF* (both described above).

BT1-6 are F4 RILs that were derived from the 6 earliest-flowering F2s of an approximately 90 member Bur-0 X Tul-0 population.

Bur-0 *FLC* FRI-Col was created by introgressing Bur-0 *FLC* into a FRI-Col background out to the backcross 4 generation. This line is Bur-0 at the *LIF* region.

Bur-0 *FLC* Tul-0 was created by introgressing Bur-0 *FLC* into a Tul-0 background out to the backcross 4 generation. This line is Bur-0 at the *LIF* region.

Description of segregating populations used to generate figures:

Segregating *LIF* population, FRI-Col *flc-3* background (Figure 1a): FRI-Col+Tul-0 *LIF* (described above), a backcross 9 line, was crossed to FRI-Col *flc-3* to create a backcross 10 population. From this population, an F2 recombinant containing *flc-3* and the Tul-0 *LIF* region on the same chromosome was selected. This recombinant was crossed to FRI-Col *flc-3* to create a backcross 11 F1, which served as parent of this population (FRI-Col *flc-3* background).

Segregating *LIF* population, Tul-0 *flc-3* background (Figure 1a): Tul-0 *flc-3* and Tul-0 *flc-3*+Tul-0 *LIF* (both described above) were crossed to create a segregating backcross 7 population.

Segregating *FEN1* population #1 (Figures 2a and 2b): Tul-0 *FLC* FRI-Col and FRI-Col+Tul-0 *FEN1* (both described above) were combined genetically to create a Tul-0/Tul-0 *FLC*, Tul-0/Col *FEN1* plant, which served as the parent of this population (FRI-Col background).

Segregating *FEN1* population #2 (Figures 2a and 2b): FRI-Col+Tul-0 *LIF* and FRI-Col+Tul-0 *FEN1* (both described above) were combined genetically to create a Tul-0/Tul-0 *FLC+LIF*, Tul-0/Col *FEN1* plant, which served as the parent of this population (FRI-Col background).

Segregating *FEN1* population #3 (Figures 2a and 2b): Tul-0+Col *FEN1* and Tul-0+Col *LIF* (both described above) were combined genetically to create a Col/Col *LIF*, Tul-0/Col *FEN1* plant, which served as the parent of this population (Tul-0 background).

Segregating *FEN1* population #4 (Figures 2a and 2b): This is the F2 population from which Tul-0+Col *FEN1* (described above) was derived (Tul-0 background).

Segregating Col *FLC*/*flc-3*, FRI-Col background (Figure 4a): FRI-Col *flc-3* was crossed to FRI-Col to generate an F1, the parent of this population.

Segregating Col *FLC*/*flc-3*, FRI-Col+Tul-0 *FEN1* background (Figure 4a): FRI-Col *flc-3* was crossed to FRI-Col+Tul-0 *FEN1* to generate an F1, the parent of this population.

Segregating Col *FLC*/*flc-3*, Tul-0 background (Figure 4a): Tul-0 *flc-3* was crossed to Col *FLC* Tul-0 to generate an F1, the parent of this population. This population is Col at the *LIF* region.

Segregating *FRI*/*fri* population, Col background (Figure 5a): FRI-Col was crossed to Col to create an F1, from which this population was derived.

Segregating *FRI*/*fri* population, Tul-0 background (Figure 5a): This is the F2 population from which *fri* Tul-0 (described above) was derived.

Growth conditions:

As described in chapter 2, this work. Most experiments were conducted under long day conditions (16 hours light/8 hours dark); one experiment was conducted under short day conditions (8 hours light/16 hours dark).

Flowering time measurements:

As described in chapter 2, this work. Some experiments were terminated before the latest subset had flowered. In these cases, a leaf number (90-120) was simply assigned to the latest subset. This value is specified in the figure legends of the data associated with these experiments.

Genotyping and mapping primers:

flc-3: The nonfunctional *flc-3* allele was distinguished from Tul-0 and Col *FLC* using a deletion flanked by primers cccgacgaagaaaaagtagataggc and acaaacacagaaccgagaaacaaca.

fri: The nonfunctional Col *fri* allele was distinguished from the functional FRI-Col (SF-2) and Tul-0 *FRI* alleles using primers described in chapter 2 of this work.

FLC: Col, L*er*, and Tul-0 *FLC* primers as described in chapter 2.

Da (1)-12 *FLC*: During introgression, Da (1)-12 *FLC* was distinguished from other *FLC* alleles using one primer within the Da (1)-12 *FLC* retroelement (tctccggaagacaagaaaagcacc) and one primer outside of the element (attaagtgaagttttgtcaaaattgtt).

LIF segregating populations: In Figure 1a, an indel at 4.6 Mb on Chromosome V, flanked by primers aacccaaactaaagcaatgaaaac and aatgtgcatcatagtttcagtttc, was used to distinguish Tul-0 and Col DNA. For the purpose of line construction, the *LIF* region was

defined on Chromosome V as between 4.6 and 6.8 Mb; a 6.8 Mb indel was flanked by primers ggaacttccaaatccgccgtttccg and ccagtatcgctcatgaggatcaaga.

FEN1 segregating populations: In Figure 2a, an indel at 21.2 Mb on Chromosome I, flanked by primers ccctttttgccgaatcagttctag and aaataccaatgacagtttagatgg, was used to distinguish Tul-0 and Col DNA. For the purpose of line construction, the *FEN1* region was defined on Chromosome I as between 21.2 and 24.6 Mb; a 24.6 Mb indel was flanked by primers gggatatcagaaggatccatagagg and gaatctttagccttgctacttttttc.

Search for *FEN* loci: As described in the "Col vs. Tul-0 mapping primers" section of chapter 2 Materials and Methods, with the exceptions that the 2.2 Mb marker on Chromosome V was here omitted and a 6 Mb indel flanked by primers ataattaccaccaccaaagcgtcc and acgtggcggcggcagcttcagggg was here employed.

Expression analysis:

As described in chapter 2, this work.

RESULTS

Late Independent of FLC is a Tul-0 locus on Chromosome V that delays flowering by an *FLC*-independent mechanism

The fact that Tul-0 *FLC* does not confer a late-flowering phenotype in a FRI-Col background, together with its poor expression (chapter 2, this work), suggested that Tul-0's late-flowering phenotype might be *FLC*-independent. In order to examine this model, we introgressed *flc-3*, a nonfunctional allele obtained through mutagenesis of FRI-Col (Michaels and Amasino 1999), into a Tul-0 background. At each backcross generation, we found that all *flc*-

3/flc-3 F2 plants flowered as early as FRI-Col *flc-3*, which seemed to indicate that, contrary to our hypothesis, Tul-0's late-flowering phenotype was entirely *FLC*-dependent. Parallel introgressions, in which we introduced the functional Col *FLC* into a Tul-0 background, created extremely late-flowering plants (data not shown), indicating that the earliness of Tul-0 *flc-3/flc-3* plants was due to the *flc-3* allele and not per se to the replacement of regions of Tul-0 Chromosome V with the corresponding regions from FRI-Col.

However, our results did not preclude the possibility that Tul-0 loci present in genomic regions that had remained FRI-Col in our advanced introgression lines—for instance, Tul-0 loci closely linked to *FLC* itself—might confer an *FLC*-independent phenotype. In our early-flowering backcross 7 Tul-0 *flc-3* line, the junction between Tul-0 and FRI-Col DNA on Chromosome V lay between 5.1 Mb and 5.3 Mb, approximately 2 Mb below *flc-3*, which was present at 3.2 Mb. In order to examine whether loci in this *FLC*-linked region might confer an *FLC*-independent phenotype, we screened for recombinants within this Chromosome V region and obtained three new lines that were Col *flc-3* (3.2 Mb) but Tul-0 at a marker at 4.6 Mb. Each of these three new lines flowered approximately 10 leaves later than the early-flowering Tul-0 *flc-3* control, indicating the presence of an *FLC*-independent flowering locus between 3.2 and 5.3 Mb, which we named *Late Independent of FLC (LIF).*

In order to control for maternal effects and for the contributions of unlinked loci that may not have been eliminated during introgression, we decided to examine the phenotypic effect of *LIF* in a segregating population descended from a single heterozygous parent. Further, in order to determine whether *LIF* acted independently or whether its action instead required additional Tul-0 loci, we created two segregating populations: the first in a FRI-Col *flc-3* background (backcross 11) and the second in Tul-0 *flc-3* background (backcross 7).

We planted more than 50 members of each of these populations. For each plant, we determined both the total number of leaves formed at flowering and the genotype at a PCR marker at 4.6 Mb on Chromosome V (Figure 1a). In both populations, Tul-0/Tul-0 homozygotes flowered later than Col/Col homozygotes, confirming that *LIF* is located on the top of Chromosome V (Figure 1a). In both populations, Col/Tul-0 heterozygotes also formed a distinct population, flowering earlier than Tul-0/Tul-0 homozygotes but later than Col/Col homozygotes, which indicated that *LIF* acts semi-dominantly. Finally, *LIF* exerted approximately the same phenotype in both a Tul-0 background and in a FRI-Col background, suggesting that *LIF* action does not depend upon the presence of unlinked Tul-0 loci (Figure 1a).

One classical flowering time pathway, mutations in which alter flowering time independent of *FLC*, is the photoperiod pathway (Michaels 2008). Variation in the photoperiod response has been demonstrated in natural populations, and natural variants of components of the day length sensing machinery have been shown to contribute to natural variation in flowering time (Giakountis et al. 2009; Aukerman et al. 1997; El-Assal et al. 2001; Balasubramanian et al. 2006). Complete abrogation of the photoperiod pathway through the loss of long day floral promoters, such as *CONSTANS* (*CO*) and *GIGANTEA* (*GI*), creates a photoperiod-blind plant that flowers late in long days but flowers no later in short days (Koornneef et al. 1991; Suárez-López et al. 2001; Park et al. 1999). Interestingly, *CO* itself is located in the *LIF* region, raising the possibility that *LIF* might be a natural variant of *CO* itself. However, sequencing of Tul-0 *CO* revealed no obvious loss of function mutations, including no changes in the coding region (data not shown).

To examine whether the photoperiodic response might be altered in a *LIF*-containing line, we grew *flc-3* lines with and without *LIF* both in long days and in short days (Figure 1b).

All lines, including the *LIF*-containing line, flowered substantially later in short days than in long days, indicating that, unlike loss of function alleles of *CO* and *GI*, Tul-0 *LIF* does not eliminate the photoperiodic response (Koornneef et al. 1991). Interestingly, FRI-Col *flc*-3 flowered later than the Tul-0 *flc-3* lines in short days, a phenotype not apparent in long days (Figure 1b). However, since the Tul-0 *flc-3* lines both with and without *LIF* behaved the same way, this comparative early flowering in short days is not caused by the *LIF* locus. Instead, an unlinked, non-*LIF* Tul-0 locus is likely responsible. Whether *LIF* acts through the photoperiod pathway—perhaps through a mechanism more subtle than the classical *co* and *gi* mutations—therefore remains unclear.

FLC-enhancer **loci delay flowering by an** ***FLC*****-dependent mechanism that is accompanied by an upregulation of** ***FLC*** **expression**

Because *LIF*, the only *FLC*-independent locus we had been able to identify, did not appear sufficient to account for the entirety of Tul-0's late-flowering phenotype, we reasoned that this phenotype must be in part *FLC*-dependent. Our earlier demonstration that Tul-0 *FLC* is functional (chapter 2, this work) is consistent with this model.

In order to identify Tul-0 loci involved in the *FLC*-dependent delay of flowering, for which we proposed the name *FLC-Enhancer*s (*FEN*s), it was first necessary to create a mapping population. We reasoned that in a FRI-Col × Tul-0 F2 population it would be difficult to identify *FEN* loci because the strong Col *FLC* would confer a late-flowering phenotype even in the absence of such loci. We therefore crossed an early-flowering Tul-0 *FLC* FRI-Col line back to the late-flowering Tul-0 parent, creating an F1 that was fixed at the weak Tul-0 *FLC* but FRI-Col/Tul-0 heterozygous at the remainder of the genome. The offspring of this F1 plant served as

our initial mapping population. We planted more than 250 members of this population, and, since early-flowering appeared to be a recessive trait, we selected the 24 earliest flowering plants. Using multiple markers on each chromosome, we found that FRI-Col alleles were enriched both at the top of Chromosome V, peaking at a marker at 6 Mb, and on Chromosome I, peaking at a marker at 21.2 Mb. In order to determine whether this enrichment was in fact correlated with the flowering phenotype or whether it was instead the result of some unrelated selection bias, we also genotyped the 24 latest flowering plants. In these latest plants, we found an enrichment of Tul-0 alleles at both 6 Mb on Chromosome V and at 21.2 Mb on Chromosome I, confirming a correlation between flowering phenotype and these two genomic regions.

It was expected that the effect of *LIF* would be apparent in this population, and the appearance of the Chromosome V peak within the *LIF* region seemed to confirm this prediction. We therefore focused our subsequent work on the peak at 21.2 Mb on Chromosome I, which we named *FEN1*. In order to confirm the effect of *FEN1* and to examine its behavior in both a Tul-0 and FRI-Col background, we introgressed both the Tul-0 *FEN1* region into a FRI-Col background and the Col *FEN1* region into a Tul-0 background, creating two NILs: FRI-Col+Tul-0 *FEN1* and Tul-0+Col *FEN1*. In order to examine the effect of Tul-0 *FEN1* upon the weak Tul-0 *FLC* rather than upon the strong Col *FLC*, we crossed FRI-Col+Tul-0 *FEN1* to the early flowering Tul-0 *FLC* FRI-Col, and then selected a plant fixed for Tul-0 *FLC* but heterozygous for the *FEN1* region. In addition, in order to test whether *LIF* might be required for *FEN* function, we also combined our original *FEN1* introgression NILs both with Tul-0+Col *LIF*, a NIL containing Col *LIF* in a Tul-0 background, and with Tul-0 *FLC* FRI-Col+Tul-0 *LIF*, a NIL containing Tul-0 *FLC* and Tul-0 *LIF* in a FRI-Col background. Overall, we created four

populations, each of which was fixed at Tul-0 *FLC* but segregating at the *FEN1* region (Figure 2a).

We planted more than 50 members of each population. For each plant, we determined both the total number of leaves formed at flowering and the genotype at a PCR marker at 21.2 Mb on Chromosome I. In each of these populations, Tul-0/Tul-0 homozygotes flowered later than Col/Col homozygotes, confirming that *FEN1* is located at the bottom of Chromosome I (Figure 2b). In addition, the fact that *FEN1* exerted a phenotype in all four populations also indicated that Tul-0 *FEN1* is active in both a Tul-0 and a FRI-Col background and that its action does not require the Tul-0 *LIF* region (Figure 2b). In addition, in these populations, the flowering time of plants heterozygous at *FEN1* was intermediate to that of Col homozygous and of Tul-0 homozygous plants, suggesting that *FEN1*, like *LIF*, acts semi-dominantly. It should be noted, however, that in populations 1, 3, and 4, the distinction between the heterozygote and homozygote classes, although statistically significant, was slight ($p<.05$, Student's t-test) (Figure 2b).

By comparing the behavior of population 2 with that of population 4, it was apparent that the addition of both *LIF* and *FEN1* did not make the Tul-0 *FLC* FRI-Col NIL as late-flowering as Tul-0; similarly, by comparing the behavior of population 1 with population 3, it was apparent that the loss of both *LIF* and *FEN1* did not make Tul-0 as early-flowering as the Tul-0 *FLC* FRI-Col NIL (Figure 2b). These observations together indicated that loci in addition to *LIF* and *FEN1* likely also contribute to Tul-0's late-flowering phenotype. Our earlier failure to define such loci in an *flc-3* background further suggested that these additional loci, like *FEN1*, might act via an *FLC*-dependent mechanism.

We next examined whether, in Tul-0, *FEN* loci might act by elevating *FLC* mRNA levels, as does the classical *FLC*-enhancer, *FRI* (Michaels and Amasino 1999). By quantitative PCR, we determined the expression of *FLC* in three lines: (1) Tul-0 *FLC* FRI-Col, (2) Tul-0 *FLC* FRI-Col+Tul-0 *FEN1*, a line derived from the segregating *FEN* population 1, which contained both Tul-0 *FLC* FRI-Col and the Tul-0 *FEN1* region in a FRI-Col background, and (3) Tul-0 itself (Figure 2c). We found that, as compared to the early-flowering Tul-0 *FLC* FRI-Col line, *FLC* expression is elevated by the addition of Tul-0 *FEN1* but is still further elevated in the native Tul-0 background. These results were consistent with a model in which *FEN1* delays flowering at least in part by elevating *FLC* expression. These results are also consistent with the existence of at least one additional *FEN* locus, which also acts by elevating *FLC* expression. A comparison of the flowering behavior of the *FEN1* segregating populations 1 and 3 further suggested that neither *FEN1* nor the hypothesized additional *FEN* locus requires the presence of the other in order to exert a phenotypic effect: first, in population 1, *FEN1* delays flowering time presumably in the absence of additional *FEN* loci; second: Col homozygotes from population 3, which lack *FEN1* but presumably contain additional *FEN* loci, flower later than Col homozygotes from population 1, which do not contain any *FEN* loci (Figure 2b).

The action of *FLC*-enhancer loci is not specific to the Tul-0 *FLC* allele

The fact that the flowering time of Tul-0 *FLC* FRI-Col NILs is delayed by mutations in some but not all members of the autonomous pathway (chapter 2, this work) suggested that Tul-0 *FLC* is unique in at least one respect. It was unclear whether this uniqueness might also underlie Tul-0's response to the Tul-0 *FEN* loci. To examine this question, we created new lines containing various additional *FLC* alleles, including the weak Da (1)-12 *FLC* and the weak L*er* *FLC*—which contain a retrotransposon and a DNA transposon, respectively, at different

positions in the first intron (Figure 3a)—and the strong Col *FLC*, which does not contain a transposon (Michaels et al. 2003).

We introgressed Da (1)-12 *FLC* into both a FRI-Col and a Tul-0 background, creating two new NILs: Da *FLC* FRI-Col and Da *FLC* Tul-0. In Da *FLC* Tul-0, the Chromosome V junction between Da (1)-12 *FLC* and Tul-0 sequence appeared to be below the *LIF* region (data not shown), suggesting that any phenotype was likely not due to *LIF*. We determined both the flowering time (Figure 3b) and the relative *FLC* expression (Figure 3c) of both Da *FLC* lines, and found that, in fact, loci from the Tul-0 background both delay flowering and elevate Da *FLC* expression with reference to the Da *FLC* FRI-Col control (Figures 3b and 3c).

We also introgressed L*er FLC* into a Tul-0 background, creating a new NIL: L*er FLC* Tul-0. In this line, the Chromosome V junction between L*er* and Tul-0 sequence appeared to be above *LIF* (data not shown), indicating that *LIF* likely would contribute to its flowering phenotype. In addition, in order to examine specifically the effect of *FEN1* on L*er FLC*, we genetically introduced Tul-0 *FEN1* to the L*er FLC* FRI-Col NIL, creating a new NIL: L*er FLC* FRI-Col+Tul-0 *FEN1*. We then determined both the flowering time (Figure 3d) and relative *FLC* expression (Figure 3e) of three lines: (1) L*er FLC* FRI-Col, (2) L*er FLC* FRI-Col+Tul-0 *FEN1*, and (3) L*er FLC* Tul-0. Reminiscent of earlier results using Tul-0 *FLC* lines, the flowering time of the L*er FLC* lines was delayed by addition of the Tul-0 *FEN1* region and still further delayed in a Tul-0 background (Figure 3d). Although *LIF* likely did contribute to the late flowering of the L*er FLC* Tul-0 NIL, it should be noted that, while *LIF* exerts an approximately 10-leaf delay, the difference in the flowering time of L*er FLC* FRI-Col+Tul-0 *FEN1* and L*er FLC* Tul-0 lines is more than 60 leaves, suggesting an additional phenotype contribution from non-*LIF* loci (cf. Figures 1a and 3d). In addition, quantitative PCR showed that L*er FLC*

expression was elevated by the addition of the Tul-0 *FEN1* region and still further elevated in a Tul-0 background, consistent with contributions from *FEN1* and from additional *FEN* loci (Figure 3e).

Finally, we also examined the effect of Tul-0 *FEN* loci on the strong Col *FLC*. Since FRI-Col is already very late-flowering, it was reasoned that it would be difficult, in FRI-Col, to detect any further lateness caused by the addition of Tul-0 *FEN* loci. Since the effect of *FLC* on flowering time is dosage dependent (Lee et al. 1994), we decided to examine this question in plants containing only one functional copy of Col *FLC*, in which flowering time would be proportionally reduced. To this end, we created three populations segregating for Col *FLC* and the nonfunctional *flc-3* allele. The first population was in a FRI-Col background, the second in a FRI-Col background with Tul-0 *FEN1*, and the third in a Tul-0 background. In this third population, the Chromosome V junctions between Col and Tul-0 sequence both appeared to be below the *LIF* region, suggesting that, in this population, any flowering phenotype would not be due to *LIF*.

We planted more than 25 members of each population and for each plant determined both the leaf number at flowering and the genotype at *FLC* (Figure 4a). There was no significant difference in the flowering time of *flc-3*/*flc-3* plants among the three populations, confirming that *FEN* loci cannot delay flowering without a functional *FLC*. *FLC*/*flc-3* heterozygotes from the Tul-0 background flowered later than heterozygotes from the Tul-0 *FEN1* population, which in turn flowered later than heterozygotes from the FRI-Col control population, consistent with a flowering delay exerted both by *FEN1* and by an additional, unlinked *FEN* locus (Figure 4a). Conversely, although *FLC/FLC* homozygotes from the Tul-0 population did flower later than *FLC/FLC* homozygotes from the FRI-Col population, the difference between *FLC/FLC*

homozygotes from the FRI-Col and *FEN1* populations was not statistically significant. It is not clear why this should be the case, but it may perhaps reflect the difficulty of accurately gauging the relative lateness of very late-flowering plants.

Col *FLC* expression is also elevated by the *FEN* loci (Figure 4b). Interestingly, however, and in contrast to our results with Tul-0, Da (1)-12, and L*er FLC* lines, the expression of Col *FLC* appears to be elevated to a greater extent in a background containing only *FEN1* than in a Tul-0 background, which may contain both *FEN1* and additional *FEN* loci. This result is puzzling, and it is not consistent with our flowering time data (Figure 4a). It should, of course, be noted that the lines used to determine *FLC* expression in Figure 4b are related to but not identical to the segregating populations used to determine flowering time in Figure 4a.

Together, these results indicate that the *FEN* loci do not act specifically on Tul-0 *FLC* but act also on the weak Da (1)-12 and L*er FLC* alleles, as well as the strong Col *FLC* allele. Tul-0 *FEN*s enhance *FLC* action by a general mechanism that does not depend upon Tul-0-specific *FLC* sequence nor the presence of a transposable element in the *FLC* intron.

Weak *FLC* compensation is partially *FRI*-independent

We had already shown that Tul-0 *FLC* is responsive to *FRI* in a FRI-Col background (chapter 2, this work). Earlier work with FRI-Col × Tul-0 F2 populations, in which, prior to vernalization, early flowering mapped to Tul-0 *FLC*, not to Tul-0 *FRI*, suggested that the Tul-0 *FRI* allele is functional (data not shown). It was not however clear whether Tul-0's late-flowering phenotype depended upon *FRI*. To examine this question, we introgressed the *fri*-null allele from Col (Johanson et al. 2000) into a Tul-0 background. By this means, we created a backcross 8 population segregating Col/Tul-0 for the FRI locus at the top of Chromosome IV.

As a control we also created a line segregating *fri*/*FRI* in a Col background. We planted 36 members of each of these populations and for each plant determined both the total number of leaves formed at flowering and the genotype at *FRI* (Figure 5a). In both populations, *fri* behaved as a recessive allele, consistent with previous reports (Lee et al. 1993). However, the *fri*/*fri* subpopulation from the Tul-0 population flowered later than the *fri*/*fri* subpopulation from the FRI-Col population, indicating that Tul-0's late-flowering phenotype is in part *FRI*-independent (Figure 5a).

It was hypothesized that this *FRI*-independent lateness might result from an *FLC*-independent mechanism mediated by *LIF*. To test this idea, we created a new NIL, *fri* Tul-0 *FLC* Col+Tul-0 *LIF*, containing Tul-0 *FLC* and the Tul-0 *LIF* region in a *fri* Col background. We compared the flowering time of this new NIL to the flowering time both of *fri* Tul-0 *FLC* Col, a previously-described line containing only Tul-0 *FLC* in a *fri* Col background (chapter 2, this work), and of *fri* Tul-0, a backcross 8 line containing *fri* in a Tul-0 background (Figure 5b). The line containing *LIF* flowered later than the Tul-0 *FLC* line but earlier than the line in a Tul-0 background, indicating that, although *LIF* does delay flowering in a *fri* background, it is not responsible for the entirety of the *FRI*-independent phenotype (Figure 5b).

This result suggested that the *FEN* loci might also contribute to *FRI*-independent lateness. We compared the expression of *FLC* in the *fri* Tul-0 *FLC* Col line and in *fri* Tul-0 and found that, consistent with a role for the *FEN* loci, *FLC* is more highly expressed in a Tul-0 background (Figure 5c). The expression of *FLC* in *fri* Tul-0 is however not as high as expression in the native Tul-0 (Figure 5c), which, together with the behavior of the segregating Tul-0 population from Figure 5a, suggested that, although late flowering is in part *FRI*-independent, *FRI* itself also contributes to the phenotype. Of course, because these data were generated using

NILs, it remains possible that the reduction both of flowering time and of *FLC* expression in *fri* Tul-0 with respect to Tul-0 is due not to the loss of *FRI* but instead to the loss of a *FRI*-linked, Tul-0-specific *FEN* locus.

In addition, we examined whether, in the absence of functional *FRI*, *FEN* loci can also act on the weak L*er FLC* and the strong Col *FLC*. We created new NILs through the genetic introduction of *fri* into some of the lines employed in Figures 3d, 3e, 4a, and 4b. In these new lines, flowering time is delayed in a Tul-0 background with reference to a Col background (Figure 5d). Although some of this delay is likely due to *LIF*, this delay is also accompanied by an elevation of *FLC* expression (Figure 5e), suggesting an additional contribution from the *FEN* loci. Together, these data indicate that the action of the *FEN* loci in a *fri* background is not specific to the Tul-0 *FLC* allele.

In the Col background, *FRI*-induced late flowering is suppressed by the loss of *FLC* function, indicating that *FRI* acts entirely through *FLC* (Michaels and Amasino 2001). In order to examine whether the same was true in Tul-0, we created a new line, *fri* Tul-0 *flc-3*+Tul-0 *LIF*, by introducing a *fri* allele into Tul-0 *flc-3*+Tul-0 *LIF*. We determined the total leaf number at flowering of the line with functional *FRI* and of the line with nonfunctional *fri* (Figure 5f). The line with the nonfunctional *fri* allele flowered earlier than the line with functional *FRI*, suggesting that, in Tul-0, *FRI* exerts *FLC*-independent effects. It remains unclear what FRI's additional target(s) might be.

Late flowering is not conferred by equivalent alleles in Tul-0 and Bur-0

Previous work with the Bur-0 accession, which is late-flowering despite possessing a putative null *FLC* allele, has indicated that loci responsible for late flowering are located at the top of Chromosome V and the bottom of Chromosome I (Werner et al. 2005a). The fact that

these Bur-0 loci occupied locations similar to those of *LIF* and *FEN1* in Tul-0 raised the possibility that late flowering in Bur-0 and Tul-0 might be conferred by the same loci. In order to begin to address this question, we created a Bur-0 X Tul-0 F2 population and selected the six earliest flowering plants. We allowed these plants to self for two additional generations, creating six F4 lines, which we called BT1-BT6. We determined the total leaf number at flowering of these six lines as compared to the Tul-0 and Bur-0 parents (Figure 6a). Bur-0 flowered earlier than Tul-0, but the six BT F4 lines flowered earlier even than the Bur-0, consistent with a model in which late flowering in Tul-0 and Bur-0 is conferred by sets of alleles that are not entirely equivalent. The non-equivalency of the Tul-0 and Bur-0 alleles was also consistent with our earlier demonstration that late flowering in Tul-0 is in part *FLC*-dependent. In contrast, late flowering in Bur-0, which possesses a nonfunctional *FLC* allele, is presumably entirely *FLC*-independent. The completion of an entire Bur-0 X Tul-0 recombinant inbred line (RIL) population, which we have already initiated, may help further address these questions and enable us to evaluate, directly and on an individual basis, whether the Bur-0 and Tul-0 alleles on Chromosome V and Chromosome I are equivalent. Of course, it is important to note the possibility that Bur-0 and Tul-0 may in fact possess equivalent flowering time alleles but that this complementation may be counterbalanced by transgressive interactions amongst additional alleles, in order, in sum, to generate an early-flowering phenotype in the BT1-6 lines.

We also introduced Bur-0 *FLC* into both a FRI-Col and a Tul-0 background by introgression, in each case, out to the backcross 4 generation. For each line, we determined the total leaf number formed at flowering and found that, in contrast to our earlier work with other naturally-occurring *FLC* alleles, Bur-0 *FLC* confers the same phenotype in a FRI-Col

background and a Tul-0 background (Figure 6b). These results indicate, consistent with our earlier model, that Tul-0 *FEN* loci cannot enhance the activity of a nonfunctional *FLC* allele.

DISCUSSION

In an effort to determine what distinguishes the FRI-Col background, in which the weak Tul-0 *FLC* confers an early-flowering phenotype, from the Tul-0 background, in which the weak Tul-0 *FLC* contributes a late-flowering phenotype, we constructed multiple specialized mapping populations. Through one of these approaches, we identified *LIF*, a locus on the top of Chromosome V, and showed that *LIF* delays flowering by an *FLC*-independent mechanism in both a Tul-0 and FRI-Col background. We also identified *FEN1*, a locus on the bottom of Chromosome I, and showed that *FEN1* delays flowering by *FLC*-dependent mechanism. We also showed that *FEN1* confers this delay in both a Tul-0 and FRI-Col background and in both the presence and absence of *LIF*. In addition, we showed that *FEN1* action is accompanied by an upregulation of *FLC* expression. Our work also supports the existence of at least one additional *FEN* locus, which also acts via an *FLC*-dependent mechanism and whose action is also accompanied by an upregulation of *FLC* expression. In addition, we showed that the *FEN* loci delay flowering not only in lines containing Tul-0 *FLC* and functional *FRI* but also in lines containing L*er*, Da (1)-12, and Col *FLC*, and in lines with nonfunctional *FRI*.

This initial characterization of the mechanism by which late flowering is conferred in Tul-0 raises a point of confusion: although late flowering in Tul-0 appears in large part to be *FLC*-dependent and although the action of the Tul-0 *FEN* loci is accompanied by an upregulation of FLC expression, Tul-0 *FLC* is nonetheless poorly expressed even in a Tul-0 background. A number of models with which to account for these facts might be suggested. First, in a Tul-0

background, a given level of *FLC* mRNA may be more efficiently distributed than in a FRI-Col background, a difference that might, here, be obscured by the isolation of RNA from whole seedlings rather than specific tissues. Second, *LIF* and perhaps additional *FLC*-independent loci may make more substantial contributions to the flowering time delay than our earlier genetic tests enabled us to estimate. Third, some of the *FEN* loci may act by post-transcriptional or post-translational mechanisms in addition to transcriptional mechanisms, elevating FLC protein levels or enhancing protein action without a commensurate increase in *FLC* expression. The resolution of these questions may require the examination of tissue-specific *FLC* expression as well as the consideration of post-transcriptional and post-translational mechanisms.

Many previous studies have implicated QTL on the top of Chromosome V and on the bottom of Chromosome I as contributing to natural variation in flowering time (Kover et al. 2009; Balasubramanian et al. 2009; O'Neill et al. 2008; Simon et al. 2008; Li et al. 2006; Shindo et al. 2006; El-Lithy et al. 2004 and 2006; Loudet et al. 2002; Alonso-Blanco et al. 1998; Kuittinen et al. 1997; Clarke et al. 1995; Jansen et al. 1995; Kowalski et al. 1994). At this time it is not clear which if any of these previously described QTL might be equivalent to *LIF* or *FEN1*, particularly since, in contrast to the current study, these QTL have not been mechanistically characterized in terms of *FLC*-dependence or -independence. Additional genetic tests, including those with the Bur-0 X Tul-0 population that we have already initiated, may serve to begin to address this question even in advance of locus identification.

It is however likely that at least some of these previously described Chromosome V QTL do correspond to *FLC*, *FY*, *FRIGIDA-LIKE 1* (*FRL1*), *ENHANCER OF AG-4 2* (*HUA2*), and *PHYTOCHROME C* (*PHYC*), naturally-occurring alleles of which have been previously shown to affect flowering time (Gazzani et al. 2003; Michaels et al. 2003; Lempe et al. 2005; Werner et

al. 2005a; Adams et al. 2009; Schläppi 2006; Wang et al. 2007; Doyle et al. 2005; Balasubramanian et al. 2006). Similarly, it is likely that at least some of these previously described Chromosome I QTL correspond to *FT* and *FLOWERING LOCUS M*/*MADS AFFECTING FLOWERING 1* (*FLM*/*MAF1*) natural alleles of which have also been shown to contribute to flowering time variation (Schwartz et al. 2009; Werner et al. 2005b).

In contrast, it is unlikely that *LIF* or *FEN1*, the loci identified in the present study, correspond to any of these previously implicated genes. We have mapped *LIF* to an approximately 2 Mb region on Chromosome V between 3.2 MB and 5.3 MB, boundaries that exclude *FLC*, *FRL1*, *HUA2*, and *PHYC*. It remains formally possible that *LIF* is an *FY* allele; however, unlike *LIF*, *FY* alleles delay flowering via an *FLC*-dependent mechanism (Simpson 2004). Similarly, we have delimited *FEN1* to an approximately 12 Mb region on Chromosome I, extending from 12.2 Mb to 24.5 Mb, boundaries which exclude *FLM*. It remains formally possible that *FEN1* may be an *FT* allele; however, *FT* acts downstream of *FLC* and, in contrast to *FEN1*, a variant *FT* allele would be expected to exert an *FLC*-independent effect (Searle et al. 2006).

The *LIF* and *FEN1* regions do however contain genes that have been shown to affect flowering time in laboratory-induced mutants. The *LIF* region contains several genes that, like *LIF*, have been previously shown to affect flowering time via an *FLC*-independent mechanism, including (1) *EMBRYONIC FLOWER1* (*EMF1*) (At5g11530), which represses expression of the floral homeotic gene *AGAMOUS* (*AG*); (2) *AGAMOUS-like 15* (*AGL15*) (At5g13790), a MADS domain floral repressor; (3) *CO* (At5g15840), which elevates expression of the floral integrator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) in response to long day conditions; and (4) *EARLY FLOWERING 9* (*ELF9*) (At5g16260), which reduces the expression

of *SOC1* (Moon et al. 2003; Calonje et al. 2008; Fernandez et al. 2000; Adamczyk et al. 2007; Suárez-López et al. 2001; Valverde et al. 2004; Song et al. 2009). This region also contains *CONSTANS-LIKE1* (*COL1*) (At5g15850), which, although closely related to *CO*, has not been shown to affect flowering time (Ledger et al. 2001).

In the same way, the *FEN1* regions contains several genes that, like *FEN1*, have been previously shown to affect flowering time via an *FLC*-dependent mechanism, including (1) *HISTONE MONOUBIQUITINATION 2* (*HUB2*) (At1g55250), a ubiquitin ligase that modifies *FLC* chromatin; (2) *VERNALIZATION INDEPENDENT 5* (*VIP5*) (At1g61040), a member of the Paf1-like complex required for high *FLC* expression; and (3) *ARABIDOPSIS LYSINE-SPECIFIC HISTONE DEMETHYLASE/SWIRM DOMAIN PAO PROTEIN1/LSD1-LIKE1* (*AtLSD1/SWP1/LDL1*) (At1g62830), a relative of the autonomous pathway member *FLD* and which, like FLD, represses *FLC* expression (Cao et al. 2008; Gu et al. 2009; Xu et al. 2009; Oh et al. 2004; Jiang et al. 2007; Krichevsky et al. 2007; Spedaletti et al. 2008). It should however be noted that a simple loss of function mutation in the latter gene, *AtLSD1/SWP1/LDL1*, would also be expected to delay flowering in part through an *FLC*-independent mechanism, by causing the ectopic expression of *FWA* (Jiang et al. 2007).

It remains to be evaluated whether Tul-0 variants of any of the four candidate *LIF* genes or any of the three candidate *FEN1* genes may account, respectively, for the effects of Tul-0 *LIF* and Tul-0 *FEN1*. None of these genes, to our knowledge, has been previously demonstrated to account for natural variation in flowering time. Such a demonstration would augment our understanding not only of flowering time evolution but also of the degree to which alterations in the gene's functions might be tolerated in natural populations.

It also remains possible that the flowering time delay associated with the Tul-0 *LIF* and

FEN1 regions is caused by other genes that we have not proposed. Such genes, in turn, may not have been identified in standard mutagenesis screens because Col and other laboratory strains may possess no functional equivalents; *FRI* is a classic example of a gene that could not have been identified through the mutagenesis of the standard laboratory accessions Col and L*er* because the Col and L*er FRI* alleles are already nonfunctional (Johanson et al. 2000). *LIF* and *FEN1* may also represent novel alleles of previously characterized genes, which, in Col, serve functions unrelated to flowering time. Such a gain of function might be consistent with the fact that both *LIF* and *FEN1* appear to act semi-dominantly.

Of course, the *LIF* and *FEN1* regions are still quite large, and in order to identify the causative genes, further mapping will be required. The *LIF* mapping populations that we have constructed are well-behaved, and the prospects of identifying *LIF* through standard mapping techniques appears good. *FEN1* mapping populations, in contrast, have not behaved well, suggesting that new approaches may be required. Although every population with which we have worked shows a correlation between the Tul-0 *FEN1* region and a flowering time delay, in these populations there are always a few individual plants which behave in an unexpected manner, plants that do not represent true recombinants because, curiously, the unexpected phenotype always disappears in subsequent generations. One possible explanation for this behavior might be the involvement of epigenetic mechanisms, meaning that, in some plants, the concordance between genotype and phenotype might not be immediate but might instead require more than one a generation to become apparent. Indeed, it has been demonstrated that epigenetic differences distinguishing natural populations are widespread and that epi-alleles can persist (or even arise anew) in mixed-parent populations (Vaughn et al. 2007; Zhai et al. 2008; Johannes et al. 2009; Reinders et al. 2009; Reinders and Paszkowski 2009). Alternatively, the penetrance of

the *FEN1* phenotype may be poor for non-epigenetic reasons, and the behavior of an individual plant may simply never be an absolutely reliable indication of its genotype, even after allowing multiple generations for the establishment of a new epigenetic state. In addition, in these mapping populations, the *FEN1* heterozygote class is poorly-defined and many individual *FEN1* heterozygote plants may be either very early-flowering or very late-flowering, further complicating efforts to identify true recombinants.

One way to address these problems with the current *FEN1* mapping populations might be to convert several of these F2 populations to recombinant inbred line (RIL) populations. An F9 RIL population would have been selfed for so many generations that epigenetic instabilities, had they existed in the first generation, would likely be reduced. In addition, in a RIL population, it would be possible to plant multiple plants of the same genotype, such that the atypical behavior of a single plant would not cause a false phenotypic classification. Finally, in an advanced RIL, the majority of plants would be expected to be homozygous, reducing the confusion caused by the heterozygote *FEN1* class. Of course, creating a RIL population that would be sufficient to map a gene, not merely to define a QTL, might be a massive undertaking. Enrichment of such a population with recombinants by pre-screening prior to RIL production might make the project more feasible.

Since *LIF* and *FEN1* do not appear sufficient to account for the entirety of the Tul-0 phenotype, it also remains necessary to search for additional Tul-0 loci. To this purpose, we have initiated the production of a RIL population derived from a cross between Tul-0 and an early-flowering line, Tul-0 *FLC* in a FRI-Col background. This population is fixed for the weak Tul-0 *FLC* but segregating over all other regions of the genome. Since RIL lines are fixed, multiple experiments can be conducted using the same lines, which might enable us not only to

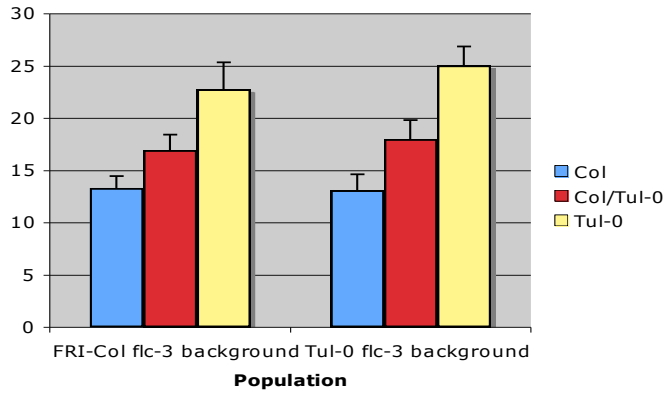
identify additional *FEN*s but also to define the relationship between late flowering in Tul-0 prior to and after vernalization.

ACKNOWLEDGMENTS

The work in this chapter was completed with the assistance of UW-Madison undergraduates Jennifer Kraninger, Libby Karn, Ellen Tangel, Curren Sharma, and Charles Calderón.

Figure 1. Effect of Tul-0 *LIF* on the flowering time of *flc-3* lines. (a) Total leaf number at flowering of offspring of Col/Tul-0 *LIF* heterozygotes in a FRI-Col *flc-3* background (left) and a Tul-0 *flc-3* background (right). In the FRI-Col *flc-3* background, offspring were divided as follows: Col *LIF* (17 plants) heterozygous *LIF* (37 plants), and Tul-0 *LIF* (15 plants). In the Tul-0 *flc-3* background, offspring were divided as follows: Col *LIF* (17 plants), heterozygous *LIF* (28 plants), and Tul-0 *LIF* (14 plants). (b) Total leaf number at flowering of FRI *flc-3*, Tul-0 *flc-3*, and Tul-0 *flc-3* +Tul-0 *LIF* under long days (LD, left) and short days (SD, right). Each data bar represents at least 11 plants.

a


30
25
20
15
10
5
0
FRI-Col flc-3 background
Tul-0 flc-3 background
Population
Col
Col/Tul-0
Tul-0

b

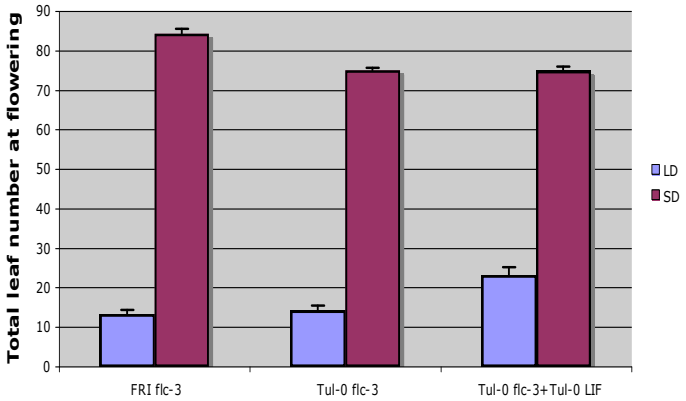
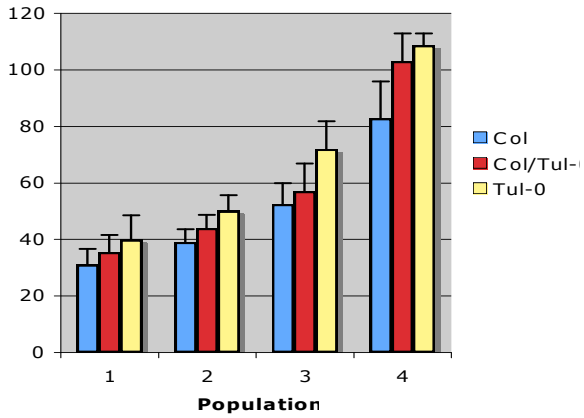

Total leaf number at flowering
90
80
70
60
50
40
30
20
10
0
FRI flc-3
Tul-0 flc-3
Tul-0 flc-3+Tul-0 LIF
LD
SD

Figure 2. Effect of Tul-0 *FEN1* on Tul-0 *FLC* lines. (a) Description of 4 populations fixed for Tul-0 *FLC* but segregating Tul-0/Col at the *FEN1* region. Here, genetic background and genotype at the *LIF* region are specified. (b) Total leaf number at flowering of Col *FEN1* (left), heterozygote *FEN1* (middle) and Tul-0 *FEN1* (right) plants from four segregating populations. Population 1 contained 15 Col (C), 26 heterozygous (H), and 19 Tul-0 (T) plants. Population 2 contained 17 C, 22 H, and 19 T plants. Population 3 contained 28 C, 26 H, and 10 T plants. Population 4 contained 20 C, 30 H, and 19 T plants. Plants that had not flowered after 5 months were assigned a value of 110 leaves. Within each population, differences among the three genotypic classes were statistically significant (Student's t-test): $p<.005$ for Col homozygotes vs. Tul-0 homozygotes; and $p<.05$ for heterozygotes vs. the two homozygote classes. (c) Relative expression of Tul-0 *FLC* in Tul-0 *FLC* FRI-Col, Tul-0 *FLC* FRI-Col+Tul-0 *FEN1*, and Tul-0.

a

Population	Background	LIF region
1	FRI-Col	Col
2	FRI-Col	Tul-0
3	Tul-0	Col
4	Tul-0	Tul-0

b



c

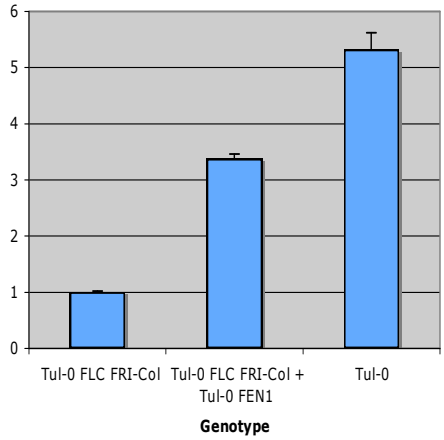
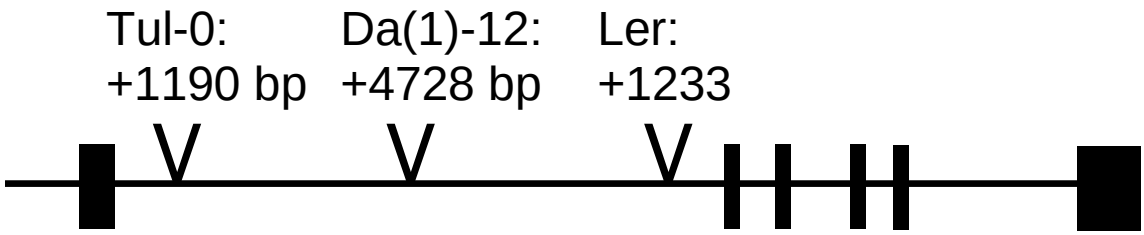
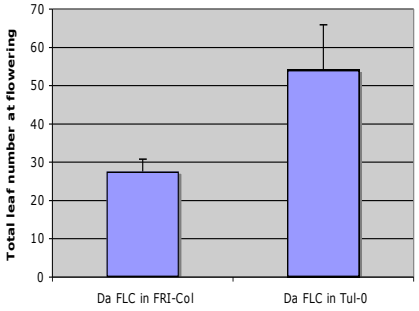


Figure 3. Effect of Tul-0 *FEN* loci on Da (1)-12 and L*er FLC* lines. (a) Location of insertions in the first *FLC* intron of Tul-0, Da (1)-12, and L*er*. Black rectangles represent *FLC* exons. Tul-0 *FLC* contains an 1190 bp insertion, consisting of an 1181 transposable element and a perfect 9 bp direct repeat of the insertion site sequence. Da (1)-12 *FLC* contains a 4728 bp insertion, consisting of a 4722 retrotransposon and an imperfect 6 bp direct repeat of the insertion site sequence. L*er FLC* contains a 1233 bp insertion, consisting of a 1224 transposable element a perfect 9 bp direct repeat of the insertion site sequence. (b) Total leaf number at flowering of Da *FLC* FRI-Col (left) and Da *FLC* Tul-0 (right). Each data bar represents at least 14 plants. (c) Relative expression of Da (1)-12 *FLC* in a FRI-Col (left) and Tul-0 (right) background. (d) Total leaf number at flowering of L*er FLC* FRI-Col (left), L*er FLC* FRI-Col+Tul-0 *FEN1* (middle) and L*er FLC* Tul-0 lines (right). Each data bar represents at least 13 plants. (e) Relative expression of L*er FLC* in FRI-Col (left), FRI-Col+Tul-0 *FEN1* (middle) and Tul-0 (right) backgrounds.

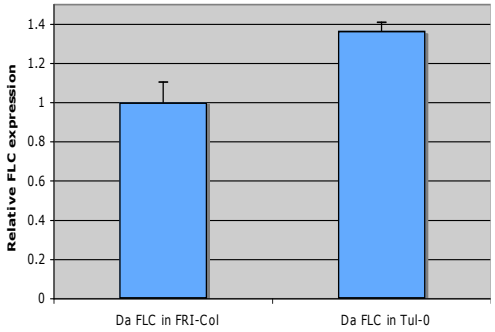
a


Tul-0:
+1190 bp
Da(1)-12:
+4728 bp
Ler:
+1233

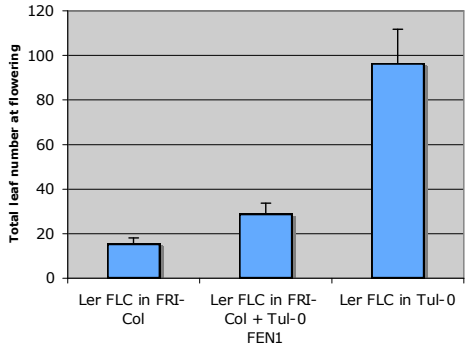
b


Total leaf number at flowering
70
60
50
40
30
20
10
0
Da FLC in FRI-Col
Da FLC in Tul-0

c


Relative FLC expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Da FLC in FRI-Col
Da FLC in Tul-0

d


Total leaf number at flowering
120
100
80
60
40
20
0
Ler FLC in FRI-Col
Ler FLC in FRI-Col + Tul-0 FEN1
Ler FLC in Tul-0

e

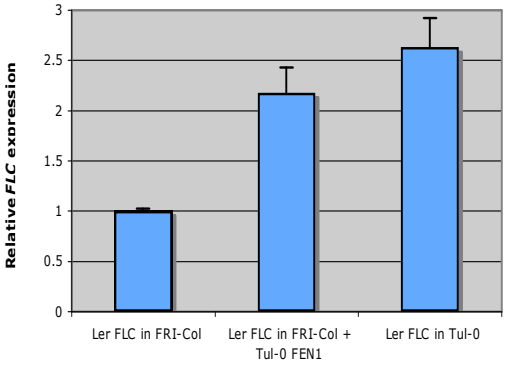
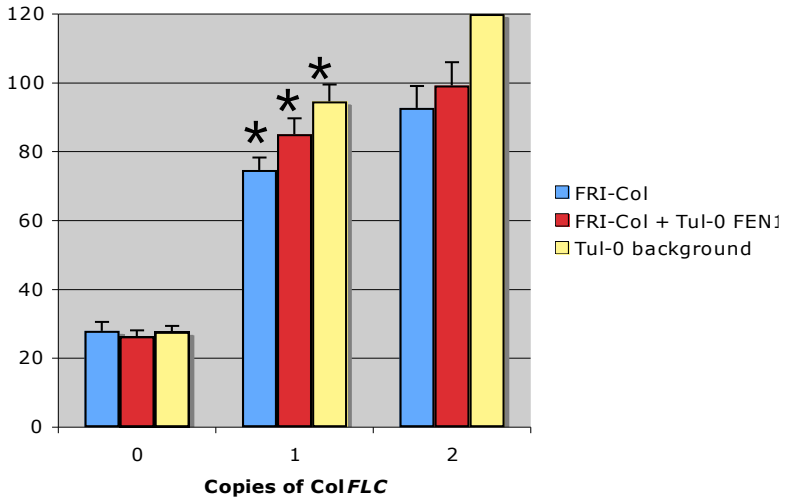

Relative FLC expression
3
2.5
2
1.5
1
0.5
0
Ler FLC in FRI-Col
Ler FLC in FRI-Col + Tul-0 FEN1
Ler FLC in Tul-0

Figure 4. Effect of Tul-0 *FEN* loci on Col *FLC* lines. (a) Total leaf number at flowering of plants with 0 (left), 1 (middle), or two (right) functional copies of Col *FLC*. Within each set of 3 bars: FRI-Col background (left), FRI-Col+Tul-0 *FEN1* background (middle), and Tul-0 background (right). Within each population, the number of plants with 0, 1, and 2 copies of functional Col *FLC* was as follows: FRI-Col background, 8, 13, and 7; FRI-Col+Tul-0 *FEN1* background, 8, 17, and 7; Tul-0 background, 10, 22, and 2. The latter two plants did not flower within the course of the experiment and were assigned a value of 120 leaves. Differences among *FLC* heterozygotes (middle set of bars, starred) in the three populations were statistically significant ($p<.005$, Student's t-test). (b) Relative expression of Col *FLC* in FRI-Col, FRI-Col+Tul-0 *FEN1* and Tul-0 *FLC* (+Tul-0 *LIF*) backgrounds.

a


120
100
80
60
40
20
0
0
1
2
Copies of Col FLC
*
*
*
FRI-Col
FRI-Col + Tul-0 FEN1
Tul-0 background

b

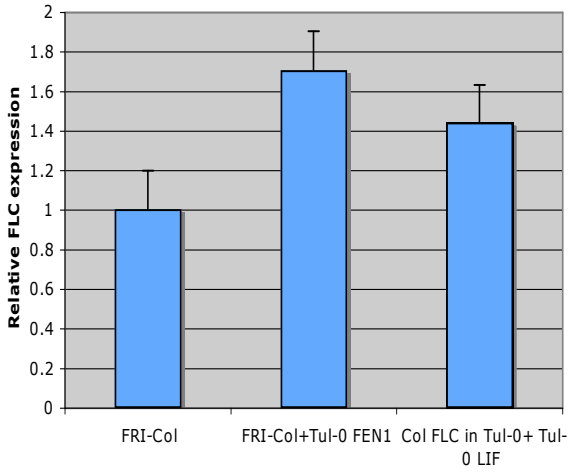
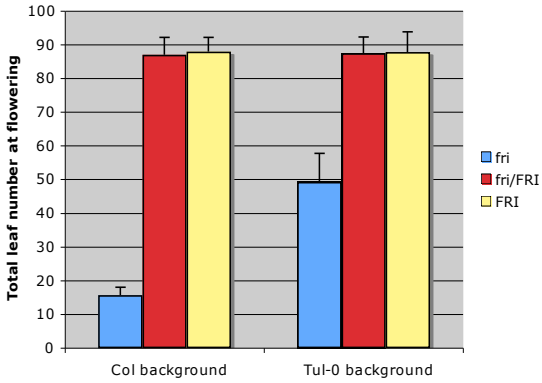
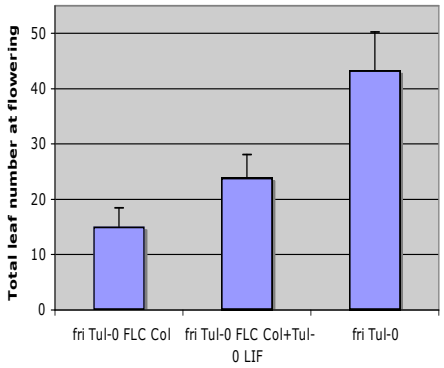

Relative FLC expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
FRI-Col
FRI-Col+Tul-0 FEN1
Col FLC in Tul-0+ Tul-0 LIF

Figure 5. Tul-0 loci delay flowering in the absence of functional *FRI*. (a) Total leaf number at flowering of plants with 0, 1, or 2 copies of functional *FRI* in a Col background (left) and in a Tul-0 background (right). Within each population, the number of plants with 0, 1, and 2 copies of functional *FRI* was as follows: Col background, 10, 16, and 10; Tul-0 background 13, 15, and 8. Plants that had not flowered after 4 months were assigned a value of 90 leaves. (b) Total leaf number at flowering of *fri* Tul-0 *FLC* Col (left), *fri* Tul-0 *FLC* Col+Tul-0 *LIF* (middle), and *fri* Tul-0 (right). Each data bar represents at least 17 plants. (c) Relative expression of Tul-0 *FLC* in *fri* Tul-0 *FLC* Col (left), *fri* Tul-0 (middle), and Tul-0 (right). (d) Total leaf number at flowering of *fri* lines with L*er FLC* and Col *FLC*. From left: *fri* L*er FLC* Col, *fri* L*er FLC* Tul-0, Col, Col+Tul-0 *FEN1*, and *fri* Col *FLC* Tul-0+Tul-0 *LIF*. Each data bar represents at least 14 plants. (e) Relative *FLC* expression of lines from Fig. 2d. (f) Total leaf number at flowering of Tul-0 *flc-3*+Tul-0 *LIF* plants with (+, left) and without (-, right) *FRI* function. Each data bar represents at least 15 plants. Stars indicate that the difference between data bars is statistically significant ($p<.005$, Student's t-test).

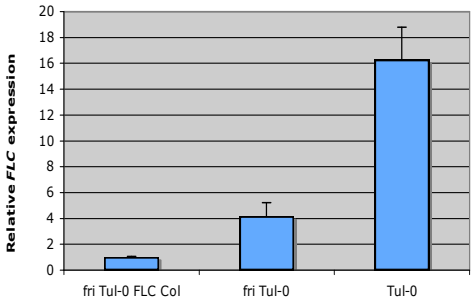
a


Total leaf number at flowering
100
90
80
70
60
50
40
30
20
10
0
Col background
Tul-0 background
fri
fri/FRI
FRI

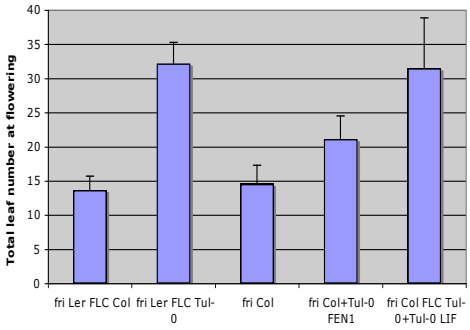
b


Total leaf number at flowering
50
40
30
20
10
0
fri Tul-0 FLC Col
fri Tul-0 FLC Col+Tul-0 LIF
fri Tul-0

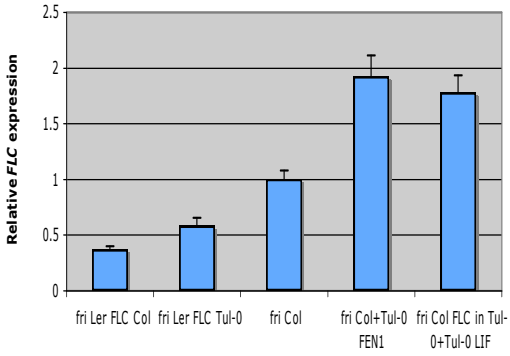
c


Relative FLC expression
20
18
16
14
12
10
8
6
4
2
0
fri Tul-0 FLC Col
fri Tul-0
Tul-0

d


Total leaf number at flowering
40
35
30
25
20
15
10
5
0
fri Ler FLC Col
fri Ler FLC Tul-0
fri Col
fri Col+Tul-0 FEN1
fri Col FLC Tul-0+Tul-0 LIF

e


Relative FLC expression
2.5
2
1.5
1
0.5
0
fri Ler FLC Col
fri Ler FLC Tul-0
fri Col
fri Col+Tul-0 FEN1
fri Col FLC in Tul-0+Tul-0 LIF

f

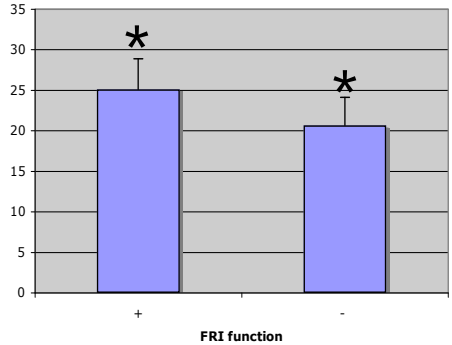
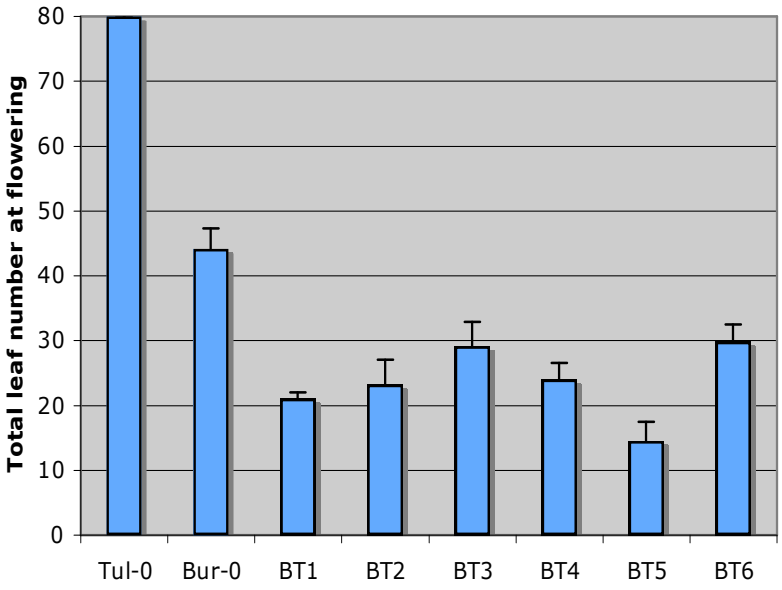
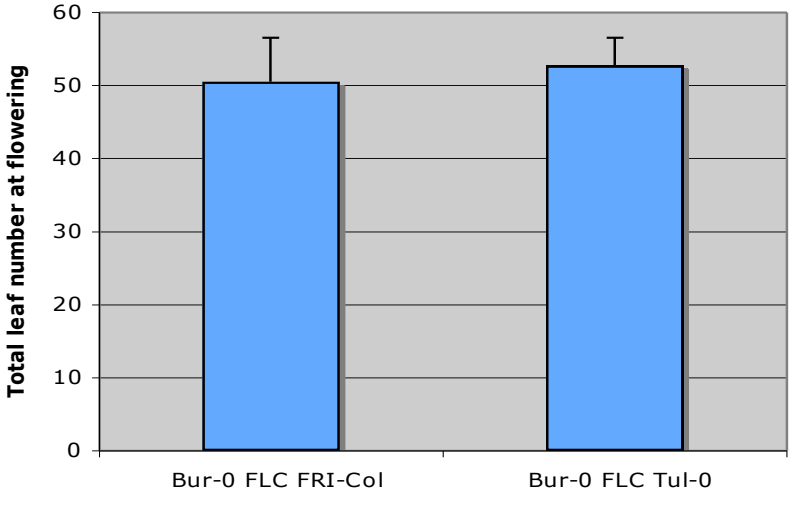

35
30
25
20
15
10
5
0
*
*
+
-
FRI function

Figure 6. Flowering behavior of Bur-0 *FLC* lines. (a) Total leaf number at flowering of Tul-0, Bur-0, and 6 derived RILs (BT1-6). Each data bar represents at least 5 plants. The experiment was terminated after 80 leaves, at which time none of the Tul-0 plants had flowered. (b) Total leaf number at flowering of Bur-0 *FLC* FRI-Col (left) and Bur-0 *FLC* Tul-0 (right). Each data bar represents 4 plants.

a


Total leaf number at flowering
80
70
60
50
40
30
20
10
0
Tul-0
Bur-0
BT1
BT2
BT3
BT4
BT5
BT6

b


Total leaf number at flowering
60
50
40
30
20
10
0
Bur-0 FLC FRI-Col
Bur-0 FLC Tul-0

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CHAPTER 4

MULTIPLE TUL-0 LOCI CONTRIBUTE TO A REDUCED SENSITIVITY TO VERNALIZATION

ABSTRACT

In many plant species, during a process called vernalization, flowering is accelerated by the experience of prolonged winter cold. In *Arabidopsis*, this acceleration is in large part mediated by a stable reduction in the expression of the floral repressor *FLOWERING LOCUS C* (*FLC*). Here, we examine a natural variant of *Arabidopsis*, Tul-0, whose flowering behavior is unusual in two respects: first, it exhibits a reduced sensitivity to vernalization and, second, it is late-flowering despite possessing a weak, poorly expressed allele of *FLC*. Using mixed parent lines, we demonstrate that these two phenotypes are genetically separable. We also show that multiple Tul-0 loci contribute to vernalization insensitivity: *REDUCED VERNALIZATION RESPONSE 1* (*RVR1*), a non-*FLC* locus, is linked to markers on the top of Chromosome V; *RVR2*, a second locus, is linked to a marker in the middle of Chromosome I. Evidence is also consistent with a contribution from an additional Chromosome V locus, which may be Tul-0 *FLC* itself. In addition, we show that, in Tul-0, vernalization-mediated silencing of Tul-0 *FLC* is not stably maintained. Unexpectedly, however, this instability in *FLC* silencing is genetically separable from vernalization-resistant late flowering.

INTRODUCTION

In plants, the transition from vegetative to reproductive growth marks an important developmental decision and, in many habitats, it is important to coordinate this transition with optimal environmental conditions. In many plant species, this requires the possession of mechanisms with which to avoid flowering in the winter and mechanisms with which to promote flowering in the spring. Accordingly, plants possess a photoperiod pathway, which, in many species, accelerates flowering during long day conditions and inhibits flowering during short day

conditions. Many species also possess a vernalization pathway, which accelerates flowering following the experience of prolonged winter cold; this is adaptive because extended winter conditions signal the imminence of spring (Chouard 1960). This requirement for prolonged cold is critical because it enables the plant to distinguish true winter from brief cold spells, which can occur in any season.

In the model plant *Arabidopsis*, vernalization accelerates flowering by reducing the expression of the floral repressor *FLOWERING LOCUS C* (*FLC*) (Sheldon et al. 1999; Michaels and Amasino 1999). The vernalization-mediated repression of *FLC*, which is accompanied by the addition of the repressive histone H3 marks H3K9 and H3K27 dimethylation to *FLC* chromatin, is mitotically stable and persists for the lifetime of the vernalized plant (Bastow et al. 2004; Sung and Amasino 2004). In the next generation, this repression is removed and *FLC* is again expressed at high levels, such that the offspring of a vernalized plant also require vernalization in order to flower rapidly (Sheldon et al. 2008; Choi et al. 2009).

Work in mutagenized populations has led to the identification of many genes required for the vernalization-mediated repression of *FLC*. One of these genes, *VERNALIZATION INSENSITIVE 3* (*VIN3*), encodes a protein containing a plant homeodomain (PHD) and a fibronectin 3 (FNIII) domain (Sung and Amasino 2004). The expression pattern of *VIN3* reflects its importance in the vernalization response: *VIN3* expression is elevated only following prolonged cold and returns again to low levels following the return of warm conditions (Sung and Amasino 2004). Additional genes required for the vernalization response include *VIN3-LIKE 1/VERNALIZATION 5* (*VIL1*/*VRN5*), a *VIN3* relative, *VERNALIZATION 1* (*VRN1*), which encodes a non-sequence specific DNA binding protein, *VERNALIZATION 2* (*VRN2*), which encodes a homolog of *Drosophila* Suppressor of Zeste 12 (Su(Z)12), and *LIKE*

HETEROCHROMATIN PROTEIN 1/*TERMINAL FLOWER 2* (*LHP1*/*TFL2*), which encodes a homolog of the chromatin binding protein HP1 in animals (Greb et al. 2007; Sung et al. 2006a and b; Levy et al. 2002; Gendall et al. 2001; Mylne et al. 2006). Unlike *VIN3*, however, *VIL1*, *VRN1*, *VRN2*, and *LHP1* are constitutively expressed. It has been proposed that the proteins encoded by some of these genes, together with additional chromatin remodeling factors, assemble into a complex that represses *FLC* expression (Wood et al. 2006; De Lucia et al. 2008). In addition, the deletion of a region in the *FLC* intron, called the vernalization response element (VRE), creates an *FLC* allele that is not stably reduced by vernalization, suggesting that this proposed vernalization complex might interact with this region (Sung et al. 2006a).

Much of what is known about the vernalization response in *Arabidopsis* has been determined in late-flowering laboratory strains: either in FRI-Col or in *Ler* lines containing mutations that elevate *FLC* expression. However, there are many natural accessions that exhibit a reduced sensitivity to vernalization with reference to these laboratory strains, and the molecular basis of this behavior remains poorly understood (Karlsson et al. 1993; Shindo et al. 2005 and 2006; Werner et al. 2005; Lempe et al. 2005). One report, examining the molecular basis for reduced vernalization sensitivity in several Swedish accessions, found that, in these accessions, *FLC* expression is initially reduced by vernalization but that this repression is not stable and *FLC* returns to high levels following the return to warm conditions (Shindo et al. 2006). This same instability in *FLC* silencing phenotype has been reported for many of the laboratory-generated vernalization insensitive mutants and for the synthetic *FLC* construct lacking the VRE (Schmitz et al. 2008; Sung et al. 2006a; Mylne et al. 2006; Sung and Amasino 2004; Gendall et al. 2001).

Here, we examine the molecular basis for the vernalization insensitivity of another accession, Tul-0, which was first collected in North America. We have previously shown that

Tul-0 exhibits a reduced sensitivity to vernalization and that it is late-flowering despite possessing a weak *FLC* allele (chapters 2 and 3, this work). Here, we show that these two phenotypes are genetically separable. We also initiate a genetic dissection of Tul-0's vernalization insensitivity and show that causative loci map to the top of Chromosome V and to the middle of Chromosome I. We also show that, in Tul-0, the expression of *VIN3* is elevated by vernalization but not to the same extent as in the FRI-Col control. In addition, in Tul-0, *FLC* expression is initially reduced by vernalization but this reduction is not stable, a result that is reminiscent of previous work with the Swedish accessions referenced above (Shindo et al. 2006). However, for reasons that may be related to the fact that Tul-0 *FLC* is weak (chapter 2) and to the fact that *FLC* enhancer loci are present in the Tul-0 background (chapter 3), this instability in *FLC* silencing is genetically separable from reduced vernalization sensitivity. A model in which Tul-0 *FLC* itself may contribute to reduced vernalization sensitivity is discussed.

MATERIALS AND METHODS

Plant Materials:

Tul-0, Pna-17, Col, and FRI-Col are as described in chapter 2.

FRI-Col *flc-3* is as described in chapter 3.

The weak Tul-0 *FLC* and weak L*er FLC* alleles are as described in chapter 2.

The weak Da (1)-12 *FLC* allele is as described in chapter 3.

Line description:

Introgression lines were constructed as described in chapter 2.

L*er FLC* FRI-Col is as described in chapter 2. L*er FLC* Tul-0, Da (1)-12 *FLC* Tul-0, and Da (1)-12 *FLC* FRI-Col are as described in chapter 3.

LT10, LT15, LT24, LT47 are F5 RILs containing L*er FLC* derived from a L*er* X Tul-0 F2 population. The 5 F2 progenitors of these 5 lines were selected from an approximately 90 member population based upon the fact that they were late-flowering and yet also homozygous for the weak L*er FLC*.

DT1 was created through the introgression of Da (1)-12 *FLC* into a Tul-0 background out to the backcross 3 generation.

Tul-0 *LIF* +, - lines: Origins of Tul-0 *flc-3* and of Tul-0 *flc-3*+Tul-0 *LIF* are described in chapter 3. FRI *flc-3*+Tul-0 *LIF* is a backcross 10 line selected from a FRI-Col *flc-3* segregating *LIF* population, which is described in chapter 3. Generation of Tul-0 *FLC* FRI-Col and of Tul-0 *FLC* FRI-Col+Tul-0 *LIF* is described in chapter 2 and chapter 3, respectively.

Col *FLC* Tul-0+Tul-0 *LIF* is as described in chapter 3.

Tul-0+Col *LIF* is as described in chapter 3.

fri Tul-0 is as described in chapter 3.

Description of segregating populations used to generate figures:

Segregating Col/Tul-0 for the top of Chromosome V in a Tul-0 background: (Figure 2a and 2b): The top half of Col Chromosome V was introgressed into a Tul-0 background out to the backcross 6 generation. The backcross 6 F1 served as the parent of this population.

Segregating population 1, Fixed *RVR1* Tul-0, segregating Col/Tul-0 for *FLC* (Figure 2c): Tul-0 was crossed to Col *FLC* Tul-0+Tul-0 *LIF* (described above), to create a backcross 7 line in a Tul-0 background.

Segregating population 2, Fixed Col *FLC*, segregating Col/Tul-0 for *RVR1* (Figure 2c): Col *FLC* Tul-0 and Col *FLC* Tul-0+Tul-0 *LIF* (both described chapter 3) were crossed and the F1 served as parent of this population (Tul-0 background).

Segregating population 3, Fixed Tul-0 *FLC*, segregating Col/Tul-0 for *RVR1* (Figure 2c): Tul-0 was crossed to Tul-0+Col *LIF* (described above), to create a backcross 7 line in a Tul-0 background.

Segregating population, Col/Tul-0 *RVR2* in a FRI-Col background (Figure 3): The middle region of Tul-0 Chromosome I was introgressed into a FRI-Col background out to the backcross 7 generation. At the final generation, a recombinant was selected specifically to exclude the Tul-0 *FLC ENHANCER 1* (*FEN1*) region (chapter 3, this work), a linked locus. This recombinant was crossed again to FRI-Col to create a backcross 8 F1, which served as the parent of this population (FRI-Col background).

Segregating population, Col/Tul-0 *RVR2* in a Tul-0 background (Figure 3): The middle of Col Chromosome I was introgressed into a Tul-0 background; a backcross 6 F1 served as the parent of this population (Tul-0 background). It was also verified that this population is Tul-0 at the Chromosome I *FEN1* region.

Growth conditions and Expression analysis:

As described in chapter 2, this work.

Vernalization:

Vernalization treatment was as described in chapter 2. All vernalization treatments were for 40 days at 4°C.

For flowering time measurements, plating of seeds was staggered such that both vernalized and non-vernalized seedlings were transferred to soil on the same day.

Conversely, for expression analysis, seeds within a given experiment were plated on the same day and tissue collection was staggered (e.g., NV, 40V, and 40V+10). Following collection, tissue was frozen in liquid nitrogen and stored at -80°C. All RNA samples within a given experiment were isolated on the same day.

Flowering time measurements:

As described in chapter 2, this work.

Many experiments were terminated before all plants had flowered and, in these cases, plants that had not flowered were assigned a value of 90 leaves. This assignment is specified in the figure legends associated with these experiments.

Chromosome V specific QTL map:

A backcross 6 population (Tul-0 background) segregating Col/Tul-0 for the top of Chromosome V (see above) was vernalized (40 days, 4°C) and 108 members were planted. All plants were genotyped using 9 Chromosome V PCR markers (see below) and for each plant the total leaf number at flowering was determined. These data were analyzed using the R program (http://www.r-project.org) (Broman et al. 2003).

PCR Primers:

Chromosome V QTL map: 9 Chromosome V indels, flanked by the following primers, were used to distinguish Tul-0 and Col DNA: 1 Mb: aaagctgcttaggctttgtgtgtg and cttcttcaatcaaagcttacctgg; 2.2 Mb: gaggacaaattacatatcttcata and cgaagagagtttgtaggagaaggg; 3.2 Mb: cctcaatcttttgttgtgaaaatcgac and ttgtgggattttcaatttcctagaggc; 4.6 Mb: ttttgggaagttttgctggaatag

and sgtacagtctaaaagcgagagtatg; 6 Mb: ataattaccaccaccaaagcgtcc and acgtggcggcggcagcttcagggg; 6.8 Mb: ggaacttccaaatccgccgtttccg and ccagtatcgctcatgaggatcaaga; 7.7 Mb: gtttcgatctctttcgatttcggc and atcccaaataacggaagttaagct; 10.4 Mb: catgggagacatttacgtgtcacg and ggagaaaatgtcactctccaccgc; 13.9 Mb: gaccaactatactctgtgttctag and gagtcattaatcttttatatgcgt.

FLC segregating population: See 3.2 Mb primers, above.

RVR1 segregating populations: See 6.8 Mb primers, above.

RVR2 segregating populations: In Figure 3, an indel at 12.2 Mb on Chromosome I was used to distinguish Tul-0 and Col DNA, using primers ctcttttctattagaaccaatgggag and aacttaaatatgagaaaacacacaatgc. During introgression, *RVR2* was defined as a region between 9.8 Mb and 12.2 Mb on Chromosome I; for this purpose, an indel at 9.8 Mb flanked by primers ggtgttaaatgcggtgttc and ttgaataatttgtaggccatg was employed.

Genomic survey mapping primers: As described in the "Col vs. Tul-0 mapping primers" section in chapter 2 of this work.

Real-Time cDNA primers: *VIN3*: tgatcggttacatcatttgctgtgag and gacagatgcagcaagaacaccttctg; *FLC* and *UBQ*: As described in chapter 2, this work.

RESULTS

Late flowering in the presence of a weak *FLC* allele is genetically separable from vernalization insensitivity

The flowering behavior of Tul-0 differs from that of the laboratory line FRI-Col in two respects: (1) Tul-0 is late-flowering despite possessing a weak *FLC* allele and (2) Tul-0 is late-flowering following 1-2 months of vernalization. Heretofore, we had examined only the genetic

basis of the first phenotype and not its relationship to the second (chapter 3, this work). Two general models describing the relationship might be proposed. First, Tul-0 loci might compensate for the weak Tul-0 *FLC* via vernalization-resistant mechanisms, in which case it would not be possible to separate the two phenotypes genetically. Second, the two phenotypes might be conferred by different sets of Tul-0 loci, in which case it would be possible to create NILs that exhibited the first phenotype but not the second.

To examine these models, we determined the flowering time of many Tul-0-derived NILs before and after 40 days of vernalization. We identified many lines with weak *FLC* alleles that were late-flowering before vernalization but early-flowering after vernalization, favoring a model in which late flowering per se and vernalization-resistant late flowering are conferred by different loci. The vernalization response of a few of the lines, four late-flowering F5 lines with the weak L*er FLC*, derived from a cross between L*er* and Tul-0 (LT10, LT15, LT24, and LT47), plus a backcross 3 introduction of the weak Da (1)-12 *FLC* into a Tul-0 background (DT1), is shown in Figure 1a.

It was of interest to examine whether the robust vernalization response of these L*er FLC* and Da (1)-12 *FLC* lines was due to the lack of Tul-0 *FLC* or to the absence of additional Tul-0 loci. Therefore, we also vernalized advanced introductions of L*er FLC* and Da *FLC* into a Tul-0 background, together with FRI-Col background controls (Figure 1b). These lines, also, exhibited a robust vernalization response, suggesting that Tul-0 *FLC* itself, or a linked Tul-0 locus, is required to produce the vernalization insensitivity phenotype.

Earlier, we had demonstrated that, consistent with a previous report, Pna-17, a North American accession that, like Tul-0, is related to Knox-10 and Pna-10, is late-flowering and vernalization-responsive (chapter 2, this work; Shindo et al. 2005; Nordborg et al. 2005). Pna-17

FLC contains the same transposon at the same position as Tul-0 *FLC* (chapter 2), and, in a recombinant inbred line (RIL) population derived from a Pna-17 × FRI-Col cross, the earliest flowering lines all contain Pna-17 *FLC* (data not shown), suggesting that Pna-17 *FLC*, like Tul-0 *FLC*, is a weak allele. The vernalization response of Pna-17, together with that of the L*er FLC* Tul-0 and Da *FLC* Tul-0 NILs and the Tul-0 and FRI-Col controls, is shown in Figure 1b. These facts together suggested that Pna-17 may possess loci required to create a late-flowering phenotype in the presence of a weak *FLC* allele but lack a separable set of loci required to produce a reduced sensitivity to vernalization.

Vernalization-specific loci map to the top of Chromosome V

These results suggested the existence of at least one Tul-0 locus, absent in Pna-17 and in the vernalization-responsive NILs, that acts specifically to delay flowering after vernalization, which we called *REDUCED VERNALIZATION RESPONSE 1* (*RVR1*). Our earlier attempts to map such a locus, in which, selecting only for phenotype, we had retained the top of Tul-0 Chromosome V following 2 backcrosses into a FRI-Col background (chapter 2), together with our failure to create vernalization-insensitive NILs by introgressing other weak *FLC* alleles into a Tul-0 background, suggested that *RVR1* was present on Chromosome V and linked to *FLC*. In order to test this hypothesis in a segregating population, we created a backcross 6 population segregating Tul-0/FRI-Col for the top half of Chromosome V into a Tul-0 background, all members of which appeared to be late-flowering prior to vernalization (data not shown). We vernalized and planted 108 members of this population, together with FRI-Col and Tul-0 controls, and for each plant determined the total number of leaves formed at flower initiation. In this segregating population, some plants were as sensitive to vernalization as FRI-Col, flowering

with fewer than 30 leaves, and others were as insensitive to vernalization as Tul-0, flowering with more than 60 leaves (Figure 2a). These results suggested that this population, although a backcross 6, was segregating for a locus sufficient to produce the entirety of the reduced vernalization sensitivity phenotype. In addition, we also genotyped each member of the population at 9 markers on the top half of Chromosome V. With these data, and using the R program (http://www.r-project.org), we generated a Chromosome V-specific QTL map (data not shown) (Broman et al. 2003). This map supported the contribution of at least one Tul-0 locus on Chromosome V, with LOD scores peaking at markers at 4.6 Mb and 6 Mb; this correlation between genotype at 6 Mb and flowering time after vernalization is presented in Figure 2a.

Previous work in several Swedish accessions has suggested that at least two Chromosome V loci contribute to vernalization-resistant late flowering, one that might be *FLC* itself and another beneath *FLC* (Shindo et al. 2006). To examine whether this might also be true in Tul-0, we screened for plants in the backcross 6 population that had undergone recombination events between *FLC* (3.2 Mb) and a marker at 4.6 Mb. We used these recombinants to generate 3 new segregating populations: (1) the first fixed for Tul-0 beneath *FLC* (in a region beneath 4.6 Mb and 6.8 Mb, which we called *RVR1*) but segregating Col/Tul-0 at *FLC* itself, (2) the second fixed for Col *FLC* but segregating at *RVR1*, and (3) the third fixed at Tul-0 *FLC* but segregating at *RVR1*. We vernalized these populations, planted at least 80 members of each, and for each plant determined the total number of leaves formed at flowering. We also genotyped each plant: in the first population, we determined the genotype at *FLC* (3.2 Mb) and, in the second and third populations, we determined the genotype at a 6.8 Mb marker on Chromosome V (Figure 2b). In all three populations, Col/Col homozygotes flowered earlier than Tul-0/Tul-0 homozygotes, again supporting the presence of causative loci on Chromosome V. However, Col/Col

homozygotes in the first population, which were Col at *FLC* but Tul-0 at *RVR1*, flowered later than Col/Col homozygotes in the second population, which were Col at both *FLC* and *RVR1*. These results are consistent with a model in which two loci contribute to vernalization insensitivity: the first is closely-linked to *FLC*, for which *FLC* itself remains one candidate, and the second, which we continued to call *RVR1*, is beneath *FLC* (Figure 2b).

RVR1 and *LIF* may be separate loci

Strikingly, *RVR1* appeared to occupy the same position as *LATE INDPENDENT OF FLC* (*LIF*), a locus that contributes to the Tul-0's late-flowering phenotype prior to vernalization (chapter 3, this work). The similarity in position suggested that *RVR1* and *LIF* might be the same locus. Although the approximately 10-leaf delay that we had documented *LIF* to exert prior to vernalization did not seem sufficient to account for the more than 20-leaf delay exerted by *RVR1* after vernalization, it remained possible that, by some unprecedented mechanism, vernalization might activate *LIF*, enabling it to delay flowering more strongly. It also remained possible that, although we had already shown that *LIF* exerts an *FLC*-independent delay (chapter 3), *LIF*'s effect might be potentiated by the presence of a functional *FLC*.

To test these ideas, we vernalized six lines, together with Tul-0 and FRI-Col controls: FRI-Col *flc-3* with and without *LIF*, Tul-0 *flc-3* with and without *LIF*, and Tul-0 *FLC* FRI-Col with and without *LIF* (Figure 2c). Prior to vernalization, all three *LIF*-containing lines, including the functional *FLC* line, delayed flowering by approximately 8-9 leaves with reference to their *LIF*-less counterparts, suggesting that *LIF* exerts no additional effect in the presence of a functional *FLC* (Figure 2c). After vernalization, flowering time was reduced in all *LIF*-containing lines, demonstrating that vernalization does not activate *LIF* and that *LIF*, unlike *RVR1*, does not exert a substantial delay following vernalization. These results therefore suggest

that *LIF* and *RVR1*, although linked, are distinct loci. It should however be noted that, after vernalization, lines with Tul-0 *LIF* do flower later than control lines lacking Tul-0 *LIF*, indicating that *LIF* does contribute to post-vernalization lateness. This is turn raises the possibility that the locus we have defined as *RVR1* may be the sum effect of multiple loci, one of which may be *LIF*.

RVR2 **maps to the middle of Chromosome I**

Heretofore, we had only examined the function of vernalization loci in a Tul-0 background. It was therefore of interest to determine whether the vernalization insensitivity exerted by *RVR1* could be transferred to a FRI-Col background. We therefore introgressed the *RVR1* region into a FRI-Col background and determined the leaf number at flowering following vernalization. This line did not exhibit a substantial delay following vernalization (data not shown), indicating either (1) that the line lacked *RVR1* or (2) that *RVR1* was not, alone, sufficient to create this phenotype and instead required the assistance of additional loci elsewhere in the genome. In order to examine this second hypothesis, we returned to the backcross 3 vernalization insensitivity mapping population that we had employed in chapter 2 of this work. Following vernalization, we selected the latest flowering plants and found that, although much of the genome was FRI-Col, the latest flowering plants were Tul-0 both at the top of Chromosome V and in the middle of Chromosome I.

To examine whether this second region, the middle of Chromosome I, might contribute to vernalization insensitivity, we introgressed the middle of Tul-0 Chromosome I into a FRI-Col background (backcross 7) and the middle of FRI-Col Chromosome I into a Tul-0 background (backcross 6). We verified that non-vernalized controls from each of these two segregating populations flowered late prior to vernalization (data not shown). In addition, we vernalized

these two segregating populations for 40 days and planted more than 70 members of each. For each plant, we determined both the total number of leaves formed at flowering and the genotype at a marker at 12.2 Mb on Chromosome I (Figure 3). In both populations, homozygous Tul-0 plants flowered later than homozygous FRI-Col plants, indicating that a locus in the middle of Chromosome I contributes to late flowering following vernalization (Figure 3). We therefore named this region *REDUCED VERNALIZATION RESPONSE 2* (*RVR2*).

Tul-0 *VIN3* expression is elevated by vernalization

In FRI-Col, the PHD finger protein finger *VIN3* and is required for the downregulation of *FLC* expression and the acceleration of flowering following vernalization. In FRI-Col, *VIN3* expression is elevated by vernalization but returns to low levels following the return of warm conditions (Sung and Amasino 2004). In order to determine whether Tul-0's reduced sensitivity to vernalization might be accompanied by differences in the expression pattern of *VIN3*, we examined the expression of *VIN3* in FRI-Col and Tul-0 by quantitative PCR both before and after 40 days of vernalization (Figure 4a). In both accessions, the 40 day vernalization treatment substantially elevated *VIN3* expression, indicating that Tul-0 *VIN3* responds to extended cold treatment. However, in Tul-0, maximum *VIN3* expression was less than half that in FRI-Col (Figure 4a). It therefore remains possible that lower absolute levels of *VIN3* underlie Tul-0's reduced response to vernalization.

Tul-0 *FLC* expression is not stably reduced by vernalization

It has been previously reported that, in a group of vernalization insensitive Swedish accessions, *FLC* expression is reduced following vernalization but, following the return to warm conditions, this reduction is not stably maintained (Shindo et al. 2006). In order to determine

whether the same might be true in Tul-0, we examined the expression of *FLC* in Tul-0 before vernalization, after 40 days of vernalization, and 10 days after the return to warm conditions (Figure 4b). As a control, we employed FRI-Col. At the same time, we also examined two additional, reciprocal lines, Col *FLC* in a Tul-0 background (Col *FLC* Tul-0+Tul-0 *LIF*) and Tul-0 *FLC* in a FRI-Col background (Tul-0 *FLC* FRI-Col). This analysis showed that, in Tul-0, *FLC* expression is reduced by vernalization. However, in contrast to FRI-Col, this reduction is not stable, and *FLC* expression is re-elevated following the return to warm conditions (Figure 4b). Taken alone, this result might suggest a model in which instability in *FLC* repression is responsible for late flowering following vernalization. However, the molecular and flowering behavior of the two artificially-constructed lines challenges this model. Although the Col *FLC* Tul-0+Tul-0 *LIF* line flowers very late following vernalization (Figure 2b: population 2, Tul-0 homozygotes), *FLC* expression is nonetheless stably repressed in this line (Figure 4b). Conversely, although Tul-0 *FLC* FRI-Col flowers very early after vernalization (Figure 2c), *FLC* is not stably reduced in this line (Figure 4b). The fact that instability in *FLC* repression is correlated not with the post-vernalization flowering phenotype but rather with *FLC* allele suggests an interesting alternative model in which cis elements in Tul-0 *FLC* cause instability in *FLC* silencing but that this instability does not necessarily affect flowering behavior following vernalization.

To properly consider these results, it may also be important to again consider the still puzzling situation regarding overall levels of Tul-0 *FLC* expression, which was discussed in chapter 3. Although the silencing of Tul-0 *FLC* is not stable after vernalization, it is also true that the absolute expression of Tul-0 *FLC* is also much lower than that of Col *FLC* prior to vernalization. In a Tul-0 background, these low *FLC* levels may be sufficient to create a late-

flowering phenotype following vernalization, for possible reasons that have been previously discussed (chapter 3). In Col *FLC* Tul-0+Tul-0 *LIF*, although *FLC* expression is stably reduced, absolute levels of *FLC* expression remain higher than those in Tul-0 prior to vernalization. This higher absolute level of expression may enable Col *FLC*, when present in a Tul-0 background, to create a late-flowering phenotype following vernalization. In contrast, in Tul-0 *FLC* FRI-Col, absolute *FLC* levels are lower even than those in Tul-0 prior to vernalization, and, although the repression of *FL*C expression in this line is not stable, absolute *FLC* expression levels remain lower than those in Tul-0 after vernalization. These low absolute levels may not be sufficient to create a late-flowering phenotype following vernalization, particularly in a FRI-Col background.

The fact that Col *FLC* is a strong allele may therefore enable it, in a Tul-0 background, to create a late-flowering phenotype following vernalization, despite the fact that its expression is stably reduced. In contrast, weak *FLC* alleles like Tul-0 may not be able to delay flowering following vernalization unless both (1) the vernalization-mediation reduction of their expression is reversed following the return to warm conditions and (2) their effects are potentiated by the action of enhancer loci in a Tul-0 background. Tul-0 *FLC* may uniquely possess elements that prevent stable vernalization-mediated *FLC* silencing and so, when present in a Tul-0 background, is able to fulfill both conditions (1) and (2). Other weak alleles, like L*er FLC* and Da (1)-12 *FLC*, may not possess these elements and so may be unable to fulfill condition (1). Such a model would be consistent with our failure to produce lines with L*er FLC* and Da (1)-12 *FLC* that are late-flowering following vernalization (Figure 1a and b).

Late flowering following vernalization is partly *FRI*-independent

We had previously shown that late flowering prior to vernalization is in part independent of the function of *FRIGIDA* (*FRI*), an *FLC*-activator (chapter 3, this work). Here, we examined

whether late flowering after vernalization might also be in part *FRI*-independent. To test this idea, we vernalized *fri* Tul-0, a line in which a null *fri* allele had been introgressed into a Tul-0 background (chapter 3), along with FRI-Col, Tul-0 and (*fri*) Col controls, for 40 days, then determined the total number of leaves formed at flowering (Figure 5). The *fri* Tul-0 line flowered later than Col prior to vernalization, confirming the *FRI*-independent lateness that we had earlier documented. The flowering of the *fri* Tul-0 line was accelerated by vernalization; however, after vernalization, *fri* Tul-0 remained later flowering than Col, indicating that late flowering after vernalization is in part *FRI*-independent.

DISCUSSION

In this work, we showed that, in the natural accession Tul-0, late flowering in the presence of a weak *FLC* allele is genetically separable from reduced vernalization sensitivity. Using additional segregating populations, we mapped loci responsible for reduced vernalization sensitivity to the top of Chromosome V, to a locus near to and perhaps identical to *FLC* and to a non-*FLC* locus, *RVR1*, located in the same region as *LIF*. In addition, we mapped another locus, *RVR2*, to the middle of Chromosome I. We also showed that the expression of *VIN3*, a vernalization-required gene, is elevated in Tul-0 following vernalization but that its absolute expression is lower than that reached in FRI-Col following the same treatment. In addition, we showed that *FLC* expression is reduced in Tul-0 following vernalization but that this expression is re-elevated following the return to warm conditions. Finally, we demonstrated that late flowering following vernalization is in part *FRI*-independent.

Our data are consistent with a role for Tul-0 *FLC* itself in creating a late-flowering phenotype following vernalization. First, a locus contributing to this phenotype maps to the

Chromosome V region containing *FLC*. Second, in both a Tul-0 and a FRI-Col background, the expression of Tul-0 *FLC* (in contrast to the expression of Col *FLC*), is not stably reduced by vernalization. Consistent with these data is a model in which Tul-0 *FLC* is incapable of recruiting vernalization machinery required for permanent silencing. Indeed, an *FLC* allele whose expression is not stably reduced by vernalization has already been produced synthetically: this allele was generated through the deletion of an *FLC* region, the VRE, between 1327 and 1645 bp (as measured from the start codon) (Sung et al. 2006a).

However, it is unlikely that the behavior of Tul-0 *FLC* is due to sequence differences in the VRE region: the only difference between the sequence of the *FLC* VRE in Col and that of the VRE in Tul-0 is a 30 bp repeat, present in Col and absent in Tul-0 (chapter 2, this work). This 30 bp indel is unlikely to be causative; both Tul-0 *FLC* and L*er FLC* possess the same 30 bp intronic deletion with reference to Col (Michaels et al. 2003) and yet L*er FLC* is stably reduced by vernalization (Gendall et al. 2001).

The most salient difference between Col *FLC* and Tul-0 *FLC* is a transposon in the first FLC intron (chapter 2, this work). This transposon is not located within the VRE but instead more than 800 bp upstream, at position 491 (chapter 2, this work). It is therefore not likely that the Tul-0 transposon disrupts the VRE directly. Still, other models, in which the transposon might disrupt essential coordination between the VRE and the *FLC* promoter, either by serving as an insulator or simply by disrupting the spacing between the VRE and the promoter, have precedent in other systems and might also be proposed (Zhao and Dean 2005; Kutach and Kadonga 2000).

The *FLC* region of course contains additional genes that have also been shown to affect flowering time, including *EARLY FLOWERING 6* (*ELF6*) (At5g04240), which represses the

expression of the floral integrator *FT* through H3K4 demethylation, and *TERMINAL FLOWER 1* (*TFL1*) (At5g03840), which prevents formation of a terminal floral meristem (Jeong et al. 2009; Noh et al. 2004; Shannon and Meeks-Wagner 1991; Ohshima et al. 1997). Whether *FLC* itself, *ELF6*, *TFL1* or another linked non-*FLC* locus contributes for the Tul-0's post-vernalization behavior awaits molecular evaluation.

It is clear that, even if Tul-0 *FLC* does contribute to the phenotype, it is not an essential contribution, but one of magnitude only. Although the fact that Tul-0 *flc-3* lines are early-flowering both before and after vernalization (Figure 2c) indicates that a functional *FLC* allele of some kind is necessary, Tul-0 *FLC* is not uniquely required. Col *FLC*, also, can create a late-flowering phenotype following vernalization, though perhaps not as effectively as Tul-0 (Figure 2b). It is still not clear whether the fact that we have been unable to create vernalization-resistant late-flowering lines that contain weak *FLC* alleles other than Tul-0 *FLC* (L*er FLC* and Da (1)-12 *FLC*) is significant.

It is also clear that a non-*FLC* Chromosome V locus, *RVR1*, contributes to late flowering following vernalization (Figure 2b). *RVR1* and *LIF* map to overlapping regions, and, despite the fact that they seem to make quantitatively different phenotypic contributions and the fact that one seems to require *FLC* function and the other does not, it remains possible that they may be the same gene. Candidate genes for *LIF* have already been proposed (chapter 3, this work). The *RVR1* region is however less well-defined than the *LIF* region and contains many additional genes that have also been previously shown to affect flowering time. *LIKE HETEROCHROMATIN PROTEIN 1* (*LHP1*), which encodes a protein required to maintain the repression of *FLC* expression following vernalization, is located within this region (Sung et al. 2006a; Mylne et al. 2006). The loss of LHP1 function would be expected to generate the same

instability in *FLC* silencing that is observed in Tul-0 (although, of course, some of our data does not support the idea that it is *RVR1* that creates this instability). However, we sequenced *LHP1* and found no changes in the coding region (data not shown).

The *RVR1* region also contains *FY*, *FRIGIDA-LIKE1* (*FRL1*), *HUA2*, and *PHYTOCHROME C* (*PHYC*), naturally-occurring alleles of which have been previously shown to affect flowering time (Adams et al. 2009; Schläppi 2006; Wang et al. 2007; Doyle et al. 2005; Balasubramanian et al. 2006). We sequenced Tul-0 *FRL1* and found, with reference to Col, a difference in the coding sequence producing a T141P change in the predicted protein. We also sequenced Tul-0 *HUA2* and found changes, that, with reference to Col, produced A514V, L1136H, Q1167K, and H1371Δ changes in the predicted HUA2 protein. Of course, no allele of either *FRL1* or *HUA2* has ever been shown to affect flowering time *after* vernalization and it is not clear whether these predicted amino acid differences would have any bearing upon such a phenotype.

Our inability to recreate Tul-0's vernalization phenotype in a FRI-Col background suggested that Chromosome V loci are not sufficient to create late flowering after vernalization. Indeed, we found that *RVR2*, a locus in the middle of Chromosome I, also contributes to late flowering following vernalization. The *RVR2* region also contains many genes that have been previously shown to affect flowering time, including *GIGANTEA* (*GI*)(At1g22770), which accelerates flowering under long day conditions, *TEMPRANILLO 1/ETHYLENE RESPONSE DNA BINDING FACTOR 1* (*TEM1*/*EDF1*) (At1g25560), which represses the expression of the floral integrator *FT*, *SUPPRESSOR OF FRIGIDA4* (*SUF4*) (At1g30970), which upregulates *FLC* expression, and *FRIGIDA LIKE 2* (*FRL2*) (At1g31814), a *FRI*-related protein that, in some accessions, upregulates *FLC* expression (Rédei 1962; Koornneef et al. 1991; Park et al. 1999;

Jung et al. 2007; Castillejo and Pelaz 2008; Kim and Michaels 2006; Kim et al. 2006; Michaels et al. 2004; Schläppi 2006). We sequenced Tul-0 *FRL2* and found three changes with reference to Col *FRL2*, which would create the following changes in the predicted FRL2 protein: S110W, P132A, and R399W. Again, it is not clear whether these differences have any bearing on flowering time after vernalization.

Whether Tul-0 *FLC* (or an *FLC*-linked locus), *RVR1*, and *RVR2* are together sufficient to create a late-flowering phenotype following vernalization has not been tested, in part because of an important technical barrier. We know, already, from our previous work, that, in order to create a late-flowering phenotype prior to vernalization, the weak Tul-0 *FLC* requires the assistance of compensatory Tul-0 loci (chapters 2 and 3, this work). Therefore, a line containing only Tul-0 *FLC* and Tul-0 vernalization-specific loci, but not compensatory loci, will flower early both before and after vernalization.

There are two possible approaches to circumvent this difficulty. First, one might work with recombinant inbred lines (RILs), in which all members have become fixed at all loci following many generations of selfing. Because such lines are almost entirely homozygous, individual lines can be characterized in terms of their response to multiple environmental conditions. In this case, the flowering time of individual lines could be determined both before and after vernalization, allowing one to distinguish, post-vernalization, early-flowering phenotypes due to the lack of compensatory loci and early-flowering phenotypes due to vernalization sensitivity. To this end, we have initiated the production of a RIL population derived from a cross between Tul-0 and FRI-Col, analysis of which may enable us to identify any loci contributing to vernalization in addition to those on Chromosome V and Chromosome I.

Another approach involves creating segregating populations in which all members are late-flowering prior to vernalization but in which the post-vernalization behavior continues to segregate. Examples of such populations are those employed in this chapter in order to delimit vernalization loci on Chromosome V; these populations were in a Tul-0 background, fixed for the compensatory loci required to create a late-flowering phenotype in the presence of the weak Tul-0 *FLC*, but segregating for vernalization loci on Chromosome V. However, since these populations were fixed Tul-0 for the majority of the genome, they were suited only to the mapping of Chromosome V loci and not to the identification of causative regions that might be located elsewhere. One approach to mapping loci elsewhere in the genome might be to employ populations that are fixed for the strong Col *FLC*, which does not require compensatory loci in order to create a late-flowering phenotype, rather than the weak Tul-0 *FLC*. Although there are indications that Tul-0 *FLC* itself contributes to the phenotype, lines with Col *FLC* in a Tul-0 background are still substantially late-flowering following vernalization (Figure 2b). Particularly intriguing is the fact that lines with Col *FLC* in a Tul-0 background are late-flowering after vernalization but a FRI-Col line into which only the *RVR1* region has been introgressed is early-flowering after vernalization. These results suggest that the *RVR1* region, although required, is not sufficient to produce Tul-0's post-vernalization phenotype. With the aim of identifying additional loci upon which *RVR1* function may depend, we have crossed the two aforementioned Col *FLC* lines: Col *FLC* in a Tul-0 background and *RVR1* region in a FRI-Col background. This population, because it is fixed for the strong Col *FLC*, may eliminate difficulties regarding early flowering prior to vernalization.

Our data regarding the molecular basis of reduced vernalization sensitivity in Tul-0 are in many respects the same as those previously reported for a group of vernalization insensitive

Swedish accessions (Shindo et al. 2006). First, as shown in chapter 2 of this work, very long vernalization treatments, in excess of 5 months, accelerate the flowering of Tul-0 and these Swedish accessions to the same degree as that of vernalization sensitive controls. In this chapter, we have documented additional similarities between Tul-0 and these Swedish accessions. First, causative loci map to similar regions: two loci on Chromosome V, one that may be *FLC* and one beneath *FLC*, and another locus on Chromosome I. Next, both Tul-0 and these Swedish accessions show the same pattern of *FLC* expression: although *FLC* expression is initially reduced by vernalization treatment, expression returns to pre-vernalization levels following the return to warm conditions. These similarities are suggestive. For this reason, it may be informative to cross Tul-0 to these Swedish accessions and to screen for vernalization sensitive offspring in the F2 populations, the appearance of which would indicate that the post-vernalization behavior of the two parent accessions was not due to the action of equivalent alleles. Again, however, this experiment would likely be complicated by the presence of the weak Tul-0 *FLC*, which would likely cause transgressive early-flowering phenotypes even in non-vernalized populations.

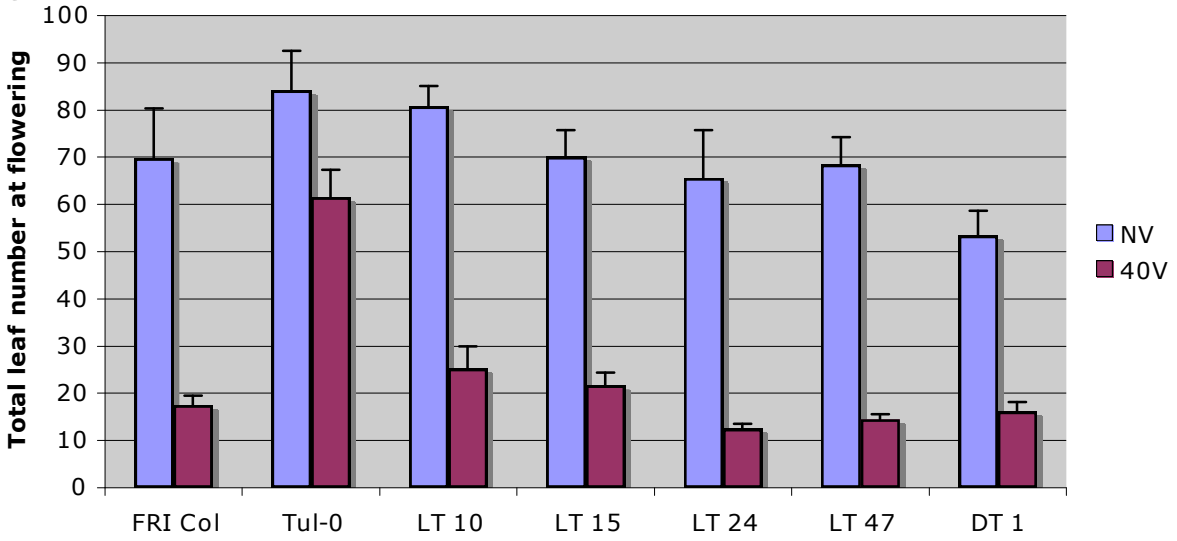
For this reason, the resolution of this question may instead await the identification of the causative alleles. If the reduced vernalization sensitivity of these distinct accessions is due to differences in the same genes, it will also be interesting to determine whether the causative alleles arose independently or whether they were instead obtained by common descent. The resolution of this question may speak to manner in which a late-flowering phenotype following vernalization might evolve, i.e., whether natural accessions may have employed multiple paths to obtain the same phenotype or, instead, whether the practicable evolutionary pathways are perhaps more limited.

ACKNOWLEDGMENTS

The work described in this chapter was completed with the assistance of UW-Madison undergraduates Jennifer Kraninger, Libby Karn, Ellen Tangel, Charles Calderón, Leah Burmeister, Curren Sharma, and Meghan Truckey.

Figure 1. Late flowering in the presence of a weak *FLC* allele is genetically separable from reduced vernalization sensitivity. (a) Total leaf number at flowering of FRI-Col, Tul-0, L*er FLC* lines (LT10, 15, 24, and 47), and a Da *FLC* line (DT1) before (NV, left) and after (40V, right) vernalization (40 days, 4°C). Each data bar represents at least 5 plants. (b) Total leaf number at flowering of Tul-0, FRI-Col, *Ler FLC* Tul-0, *Ler FLC* FRI-Col, Da *FLC* Tul-0, Da *FLC* FRI-Col, and Pna-17 before (NV, left) and after (40V, right) vernalization (40 days, 4°C). Plants that had not flowered by the end of the experiment were assigned a value of 90 leaves. Each data bar represents at least 14 plants.

a


Total leaf number at flowering
100
90
80
70
60
50
40
30
20
10
0
FRI Col
Tul-0
LT 10
LT 15
LT 24
LT 47
DT 1
NV
40V

b

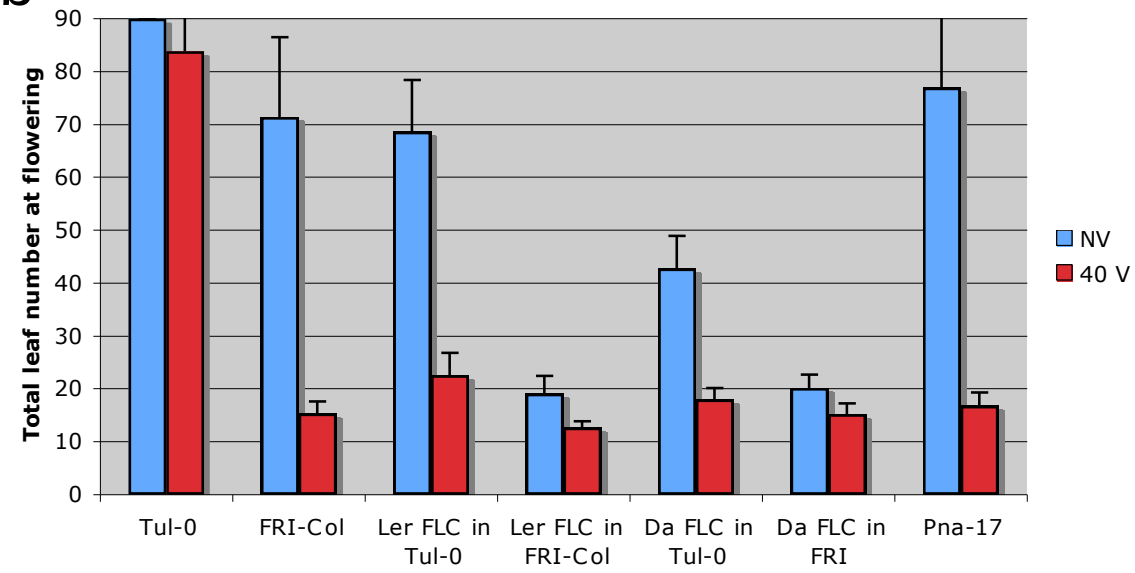

Total leaf number at flowering
90
80
70
60
50
40
30
20
10
0
Tul-0
FRI-Col
Ler FLC in Tul-0
Ler FLC in FRI-Col
Da FLC in Tul-0
Da FLC in FRI
Pna-17
NV
40 V

Figure 2. Tul-0 loci responsible for reduced vernalization sensitivity map to the top of Chromosome V. (a) Total leaf number at flowering following vernalization (40 days, 4°C) of offspring of a backcross 6 F1 segregating Col/Tul-0 for the top half of Chromosome V in a Tul-0 background. Offspring were divided into three classes based upon their genotype at a 6 Mb marker on Chromosome V: Col (left, 32 plants); heterozygous (middle, 53 plants); and Tul-0 (right, 32 plants). In the latter class, two did not flower and were assigned a value of 90 leaves. For reference: vernalized FRI-Col controls flowered with 18-25 leaves and all vernalized Tul-0 controls flowered with at least 64 leaves.

a

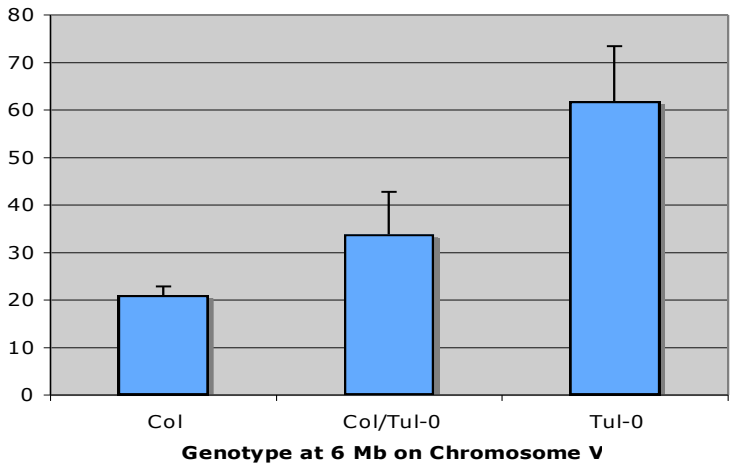
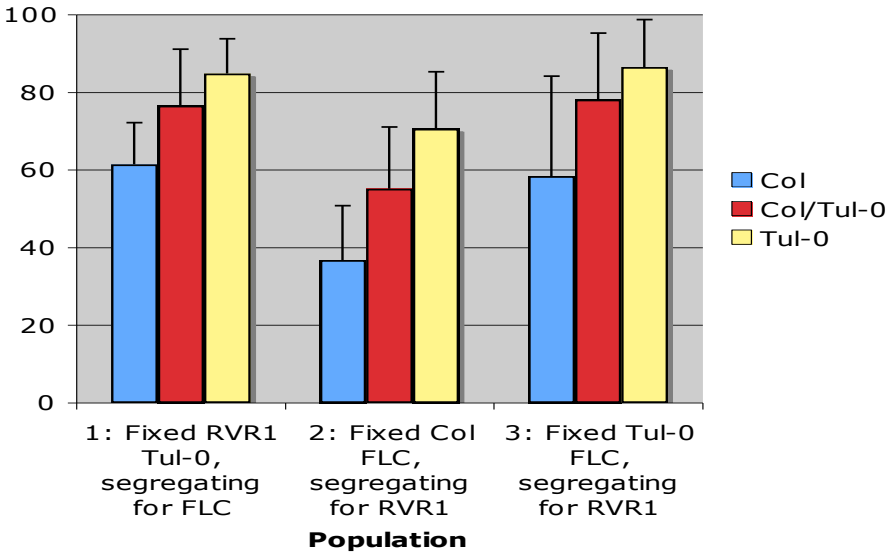

80
70
60
50
40
30
20
10
0
Col
Col/Tul-0
Tul-0
Genotype at 6 Mb on Chromosome V

Figure 2, continued. Tul-0 loci responsible for reduced vernalization sensitivity map to the top of Chromosome V. (b) Total leaf number at flowering within three populations segregating Col/Tul-0 for different regions of Chromosome V in a Tul-0 background following vernalization (40 days, 4°C). The first population (1) (left), is fixed Tul-0 at *RVR1* but segregating for *FLC* and is divided as follows: Col *FLC*, 25 plants; heterozygous *FLC*, 43 plants; and Tul-0 *FLC*, 15 plants. The second population (2) (middle) is fixed for Col *FLC* but segregating at *RVR1* (here, defined as a marker at 6.8 Mb) and is divided as follows: Col *RVR1*, 13 plants; heterozygous *RVR1*, 50 plants; and Tul-0 *RVR1*, 17 plants. The third (3) (right) is fixed at Tul-0 *FLC* but segregating at *RVR1* (here, defined as a marker at 6.8 Mb) and is divided as follows: Col *RVR1*, 22 plants; heterozygous *RVR1*, 47 plants; and Tul-0 *RVR1*, 13 plants. Plants that had not flowered at experiment termination were assigned a value of 90 leaves. Within each population, the difference between the Col and Tul-0 homozygote classes was statistically significant ($p<.005$, Student's t-test). (c) Total leaf number at flowering before (NV, left) and after (40V, right) vernalization (40 days, 4°C) of Tul-0, FRI-Col, and three pairs of lines whose members are distinguished by lacking or possessing the Tul-0 *LIF* region. *LIF* pairs, from left: FRI *flc-3* and FRI *flc-3*+Tul-0 *LIF*; Tul-0 *flc-3* and Tul-0 *flc-3*+Tul-0 *LIF*; Tul-0 *FLC* FRI-Col and Tul-0 *FLC* FRI-Col+Tul-0 *LIF*. Plants that had not flowered at experiment termination were assigned a value of 90 leaves. Each data bar represents at least 14 plants.

b


100
80
60
40
20
0
1: Fixed RVR1 Tul-0, segregating for FLC
2: Fixed Col FLC, segregating for RVR1
3: Fixed Tul-0 FLC, segregating for RVR1
Population
Col
Col/Tul-0
Tul-0

c

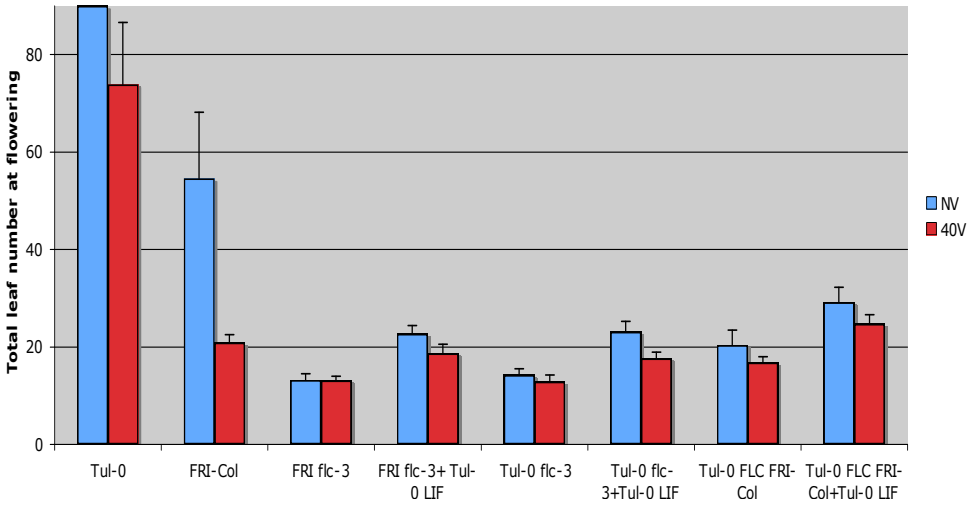

Total leaf number at flowering
80
60
40
20
0
Tul-0
FRI-Col
FRI flc-3
FRI flc-3+ Tul-0 LIF
Tul-0 flc-3
Tul-0 flc-3+Tul-0 LIF
Tul-0 FLC FRI-Col
Tul-0 FLC FRI-Col+Tul-0 LIF
NV
40V

Figure 3. A Tul-0 locus in the middle of Chromosome I contributes to late flowering after vernalization. Total leaf number at flowering after vernalization (40 days, 4°C) within two populations segregating for a locus in the middle of Chromosome I (*RVR2*, here defined as a marker at 12.2 Mb on Chromosome I). In a FRI-Col background (left), the population was divided as follows: 19 Col, 38 heterozygous, and 15 Tul-0 *RVR2* plants. In a Tul-0 background (right), the population was divided as follows: 10 Col, 41 heterozygous, and 20 Tul-0 *RVR2* plants. Within each population, the difference between the Col and Tul-0 classes (starred bars) was statistically significant ($p<.005$, Student's t-test).

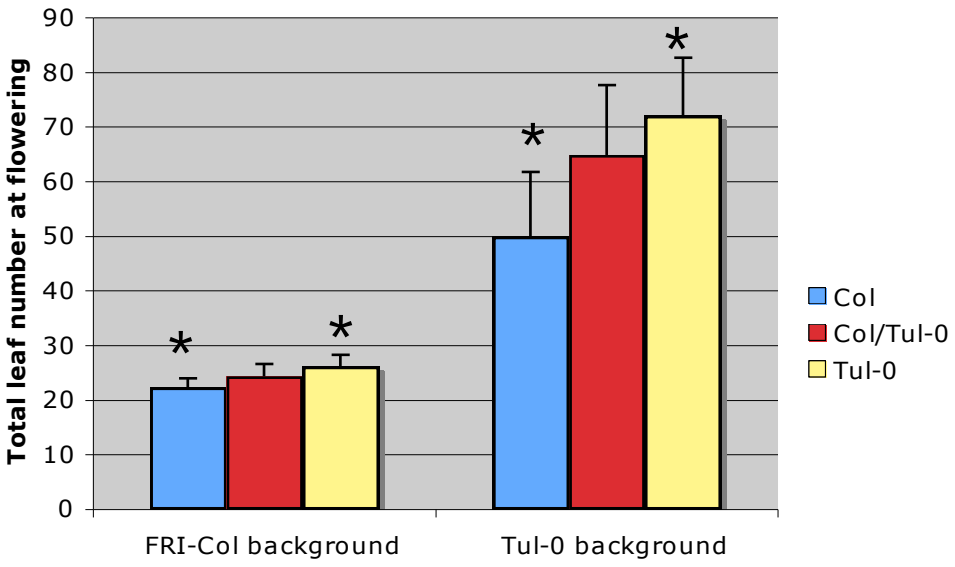
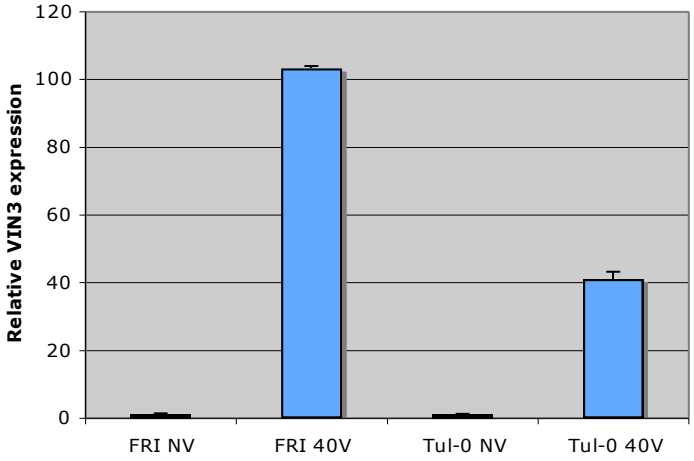

Total leaf number at flowering
90
80
70
60
50
40
30
20
10
0
*
*
*
*
FRI-Col background
Tul-0 background
Col
Col/Tul-0
Tul-0

Figure 4. (a) Relative *VIN3* expression in FRI-Col and Tul-0 before (NV) and after (40V) vernalization (40 days, 4°C). (b) Relative *FLC* expression in FRI-Col, Tul-0, and two derived lines before vernalization (NV), immediately after vernalization (40V), and 10 days after the return to warm conditions (40V+10). Vernalization treatment consisted of 40 days at 4°C.

a


Relative VIN3 expression
120
100
80
60
40
20
0
FRI NV
FRI 40V
Tul-0 NV
Tul-0 40V

b

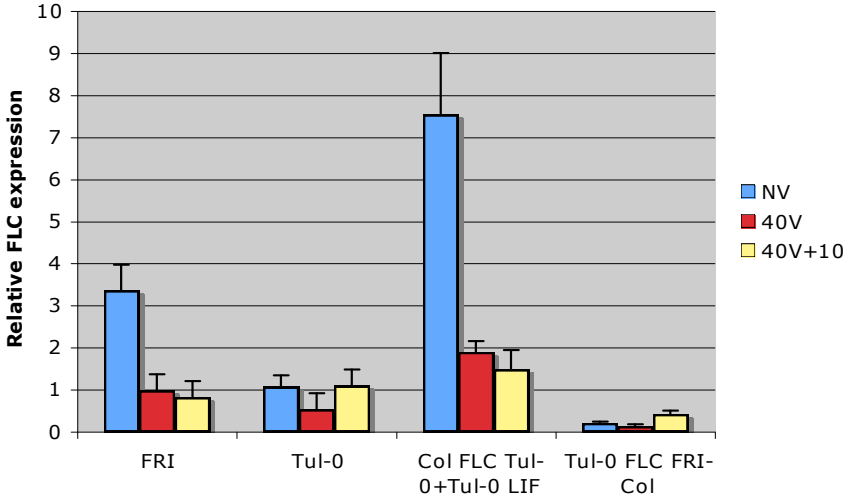
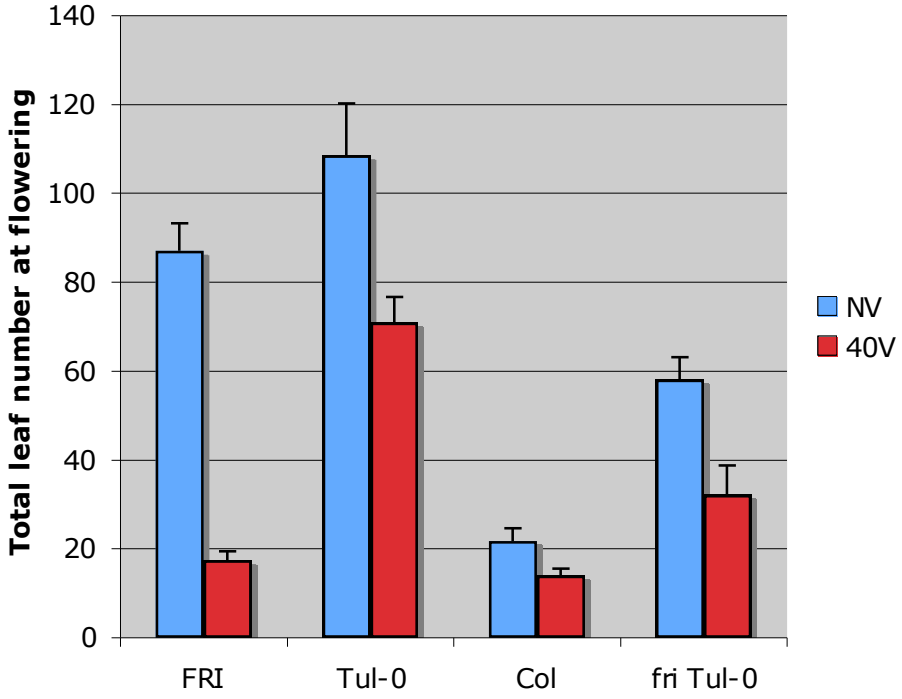

Relative FLC expression
10
9
8
7
6
5
4
3
2
1
0
FRI
Tul-0
Col FLC Tul-0+Tul-0 LIF
Tul-0 FLC FRI-Col
NV
40V
40V+10

Figure 5. Total leaf number at flowering of FRI-Col, Tul-0, Col, and *fri* Tul-0 before (NV, left) and after (40V, right) vernalization (40 days, 4°C). Each data bar represents at least 11 plants.


Total leaf number at flowering
140
120
100
80
60
40
20
0
FRI
Tul-0
Col
fri Tul-0
NV
40V

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CHAPTER 5

FINAL THOUGHTS AND FUTURE DIRECTIONS

In this work, we have examined Tul-0, a natural *Arabidopsis* accession that exhibits two unusual flowering phenotypes: first, it is late-flowering despite possessing a weak *FLC* allele; second, it exhibits a reduced sensitivity to vernalization. We have demonstrated that these two phenotypes are genetically separable, and, using NILs, have begun to dissect them. Through this dissection, we have determined that each phenotype is caused by multiple loci and that each locus, in turn, accounts for only a fraction of a given phenotype. In this way, our work is consistent with earlier models, according to which complex traits are caused by many loci, each exerting small individual effects (Weigel and Nordborg 2005; Glazier et al. 2002).

The mapping of multiple small effect loci presents particular problems not encountered in the mapping of a single large effect locus. In such cases, simple F2 populations may exhibit a wide distribution of phenotypes, but, since each individual locus accounts for only a small part of any phenotype, it can be difficult to identify the source of any one effect; conversely, when single contributing loci are moved into isogenic backgrounds, the total phenotypic variation in the new populations may be small and locus identification may remain difficult.

In this respect, the process of defining the Tul-0 *LIF* locus and of developing well-behaved *LIF* segregating populations, described in chapter 3 of this work, may be informative. *LIF* delays flowering by 8-10 leaves, accounting for only a small percentage of the phenotype of Tul-0, which flowers with more than 80 leaves. In late-flowering backgrounds such as Tul-0 and FRI-Col, *LIF* is difficult to detect. In contrast, when *LIF* is placed in early-flowering backgrounds (in this case, in *flc* mutant backgrounds, in which it nearly doubles the number of leaves formed at flowering), *LIF*'s effects are readily apparent. In order, therefore, to render small effect loci amenable to mapping, it may be necessary to remove them entirely from the parent background and to place them in new backgrounds that will magnify their relative

phenotypic effects. As with our domestication of *LIF*, this may require the use of laboratory-generated mutant alleles. Also as with the domestication of *LIF*, this may also require the patience to select for specific recombinants, sometimes, absurdly, as with *LIF*, even prior to indications that the phenotype persists in the new background.

The identification of causative genes in natural accessions as opposed to mutagenized laboratory lines in addition entails special challenges. In mutagenized populations, sequence differences between the mutant and the WT stock are frequently directly linked to the mutant phenotype; this is particularly true when mapping has already implicated the region containing the sequence difference. In contrast, in natural populations, there are often many sequence differences that are unrelated to the phenotype in question, even when, in mapping populations, these differences cosegregate with the phenotype. The ubiquity of these sequence differences has been repeatedly used to propose phylogenies explaining relationships among *Arabidopsis* accessions (Nordborg et al. 2005; El-Lithy et al. 2006; Schmid et al. 2006; Clark et al. 2007). Indeed, in the course of this work, we have sequenced many Tul-0 genes, and, in nearly every case, there has been at least one change with reference to the Col sequence, often in the coding region itself. However, it is unlikely that all or even any of the sequence differences that we have identified are phenotypically relevant; such differences are simply the consequence of the evolutionary distance between Tul-0 and Col.

Of course, this caution must also be balanced by the fact that it has been repeatedly demonstrated that small molecular differences can sometimes account for large phenotypic differences. The opposite functions of the floral promoter FT and the floral repressor TERMINAL FLOWER1 (TFL1) can be exchanged through the alteration of only one amino acid (Hanzawa et al. 2005). In a reminiscent way, two jumonji-class transcription factors,

RELATIVE OF EARLY FLOWERING 6 (REF6) and EARLY FLOWERING 6 (ELF6), although highly similar, also serve opposing functions: REF6 represses *FLC*, to accelerate flowering, and ELF6 represses *FT*, to delay flowering (Jeong et al. 2009; Noh et al. 2004). In addition, a single amino acid change in the protein CURLY LEAF (CLF) creates a novel gain of function variant that accelerates flowering (Doyle and Amasino 2009). Finally, a natural variant of CRYPTOCHROME 2 (CRY2) that creates day length insensitivity is distinguished from day length sensitive variants by only a single amino acid change (El-Assal et al. 2001). These examples highlight the possibility that, in Tul-0, the *LIF* locus, the *FEN* loci and the *RVR* loci may be distinguished from the Col equivalents by only small differences, whose phenotypic effect may not be obvious at the sequence level. Indeed, the fact that all of these Tul-0 loci appear to act semi-dominantly rather than recessively suggests that they are gain of function alleles with reference to Col, in turn suggesting that they may be due to minor sequence differences rather than obvious loss of function lesions. This, coupled with the fact that most genomic regions in Col and Tul-0 are distinguished by small differences that have no phenotypic consequences, perhaps suggests that causative loci must be fine-mapped to very small regions before the sequencing of candidate genes will be even potentially conclusive.

Of course, although mapping causative loci in natural populations does entail particular challenges, it also carries certain potential advantages with reference to mapping in mutagenized populations. Both natural and mutagenized populations may serve as tools through which to identify new genes that affect flowering time; however, an important distinction between alleles identified in natural populations and alleles identified in mutant populations is that the former may bear directly upon evolutionary questions. Defining causative alleles from natural populations may enable one to trace, on a small scale, how molecular changes lead to the

acquisition of potentially adaptive phenotypes. With additional analysis, these allelic variants might be correlated with the environmental conditions within which the natural population evolved, i.e., within which it diverged from other populations that do not exhibit the phenotype in question. Of course, in the specific case of Tul-0, inferences of environmental adaptation may be limited by the fact that *Arabidopsis* appears to be a recent introduction to the North American continent, such that its behavior may be the result of bottleneck effects rather than of true adaptation (Zwan et al. 2000; Shindo et al. 2005). However, North American loci are likely also shared by additional accessions, and the identification of causative genes in Tul-0 will likely also be informative with regard to accessions that have been associated with their environments for longer periods.

In light of the fact that the flowering time of Tul-0 and of other so-classified "vernalization insensitive" accessions is, following 5+ months of vernalization, accelerated to the same extent as the flowering time of "sensitive" control accessions, it is interesting to speculate whether true vernalization insensitivity actually exists in *Arabidopsis* (chapter 2, this work; Shindo et al. 2006). It is not clear that this phenotype can be created even through the mutagenesis of genes "required" for the vernalization response, since, in the literature, such mutants are rarely vernalized longer than required to show a phenotypic difference between the mutant and the control. For this reason, it would be interesting to conduct a long term (6 months+) vernalization time course experiment using additional vernalization insensitive accessions as well as mutants in different components of the vernalization machinery. Certain outcomes from such an experiment might imply that there are additional, perhaps redundant pathways, perhaps entirely separate from the classical vernalization pathway, that accelerate flowering after very long cold periods.

The fact that Tul-0 *FLC* lines exhibit unusual responses to the introduction of the autonomous pathway mutations *fld*, *fpa*, and *ld* but not to the introduction of *fca*, *flk*, and *fve* remains an intriguing unresolved point (chapter 2, this work). In order to distinguish between models in which this behavior is due to Tul-0 *FLC* itself and those in which the behavior is due to non-*FLC* loci that may have been retained during line construction, it will be necessary to transform autonomous pathway mutants with Tul-0 *FLC* constructs. If the results of these new experiments support models in which Tul-0 *FLC* itself is responsible for the behavior, new, chimeric *FLC* constructs might be employed in order to precisely identify the causative *FLC* region. The production and testing of these constructs might be complicated by the fact that certain alterations of Tul-0 *FLC* would likely convert it from a weak to a strong allele (reciprocally, certain alterations of Col *FLC* with Tul-0 sequence would likely convert it from a strong to a weak allele). Dramatic changes in *FLC* strength might render it difficult to compare the behavior of the new alleles with that of controls. For this reason, it might be useful to instead employ L*er FLC*, which is already weak and which exhibits expected responses to *fld*, *fpa*, *ld* (chapter 2, this work) as a control construct that would be modified by the addition of Tul-0 *FLC* sequence.

Preliminary results also suggest that the unusual responses of these Tul-0 *FLC* lines might be moderated by *FRI*, since different—although, in both cases, still curious—results were obtained in lines that in part differed due to the presence or absence of FRI function. It may therefore be useful to replicate the experiments described above in both *FRI* and *fri* backgrounds.

In light of the fact that Tul-0 *FLC* lines show unusual responses to some members of the autonomous pathway but not to others, it might also be interesting to examine the responses of lines containing other poorly-characterized *FLC* alleles. We have already introgressed Da (1)-12

FLC into a FRI-Col background (chapter 3, this work); this line could be used to create lines with autonomous pathway mutations coupled to Da (1)-12 *FLC*. Searching for novel behaviors using different natural *FLC* alleles may be a parallel method of dissecting how members the autonomous pathway or their downstream effectors interface with *FLC* in cis.

These strange responses to certain autonomous pathway mutations, which thus far appear to be specific to Tul-0 *FLC* lines, are in contrast to the action of the Tul-0 *FLC*-enhancer (*FEN*) loci, whose effects do not appear to be allele specific (chapter 3, this work). These *FEN* loci are notable because, although clearly distinct from *FRI*, whose importance in natural variation in flowering time has been well-characterized, they appear to act in a parallel manner, including through the elevation of *FLC* expression (Michaels and Amasino 1999; chapter 3, this work). Also like FRI—but unlike the SWR1 and PAF complexes, which also elevate *FLC* expression—the action of the *FEN* loci seems to be specific to *FLC*. For this reason, it may be interesting to determine whether the function of the *FEN* loci requires the presence of any of FRI's many functional partners, including FES1, SUF4, FLX, the FRL proteins, or subunits of the nuclear cap binding complex (Schmitz et al. 2005; Kim et al. 2006; Andersson et al. 2008; Michaels et al. 2004; Geraldo et al. 2009; Bezerra et al. 2004). Of course, the fact that the action of the *FEN* loci is partially but not entirely suppressed by the loss of *FRI* suggests that the results of these genetic tests may not be straightforward to interpret (chapter 3, this work). Experiments examining the effect of *fes1, suf4*, and *flx* alleles on the action of individual *FEN* loci rather than upon all of the *FEN* loci at once (e.g., in the line in which only *FEN1* has been introgressed into a FRI-Col background) may produce results that are more easily interpretable.

Perhaps still more interesting are suggestions that at least some of the *FEN* loci act via mechanisms that are unlike those of FRI. The poor expression of Tul-0 *FLC* in a Tul-0

background, coupled with indications that the late-flowering phenotype is in large part *FLC*-dependent, suggests that *FEN* loci may also operate via non-transcriptional mechanisms, perhaps at the level of FLC translation (chapters 2 and 3, this work). This possibility is interesting because it is unprecedented; the modulation of FLC levels by a translational mechanism has never been demonstrated. A mechanism in which the *FEN*s enhance FLC protein function might also be proposed. Such a mechanism has precedent, though has never been shown to affect flowering time in natural populations; in FRI-Col, FLC must complex with the MADS domain protein SVP before it can confer a late-flowering phenotype (Li et al. 2008; Lee et al. 2007; Fujiwara et al. 2008).

The identification of the genes that underlie these Tul-0 phenotypes may prove illuminating in terms of defining their mechanism of function, particularly if the Col equivalents of these genes have already been characterized. This is true not only for the *FEN* loci but also for *LIF*, which delays flowering through a yet undefined *FLC*-independent pathway, and for *RVR1* and *RVR2*, which create a reduced sensitivity to vernalization. Of the mapping populations created to identify these loci, the *LIF* populations are currently the most well-behaved, and identifying *LIF* via standard mapping techniques currently appears feasible. Conversely, fine-mapping of the *FEN* and *RVR* loci may require more creative approaches, perhaps including the production of large RIL populations.

The identification of *LIF*, the *FEN* loci, and the *RVR* loci may also enable us to begin to address specific evolutionary questions, in particular the order of acquisition of the alleles that distinguish the early-flowering Kin-0 from the late-flowering Tul-0. The possibility that Tul-0 may have descended from an early-flowering, Kin-0-like ancestor is particularly provocative because it would entail a departure from the most well-characterized pathways in flowering time

evolution. It is well-established that attenuating or loss of function mutations in *FLC* or *FRI* are single-step means through which, on many separate occasions, early-flowering accessions have evolved from late-flowering parents (Gazzani et al. 2003; Michaels et al. 2003; Lempe et al. 2005; Werner et al. 2005; Johanson et al. 2000; Le Corre et al. 2002; Shindo et al. 2005). At the present time, however, there is no evidence that, in natural populations, the acquisition of such a mutation might be anything other than a point of no return. However, the identification of *LIF* and the *FEN* loci, coupled with sequences from related accessions, might support a model in which Tul-0's ancestors were originally late-flowering but 1) became early-flowering following the acquisition of a weak *FLC* allele, and then 2) became again late-flowering following the gradual, stepwise acquisition of compensatory loci, *LIF* and the *FEN*s, which conferred late flowering in the presence of the weak *FLC*. Support for such a model would indicate that, in nature, it is also possible, albeit via a more complex and laborious route, for late-flowering *Arabidopsis* accessions to evolve from early-flowering, *FLC*-compromised ancestors.

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