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The *FLC* Locus: A Platform for Discoveries in Epigenetics and Adaptation

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Arabidopsis, *FLC*, cotranscriptional regulation, epigenetic silencing, life history variation

Abstract

Our understanding of the detailed molecular mechanisms underpinning adaptation is still poor. One example for which mechanistic understanding of regulation has converged with studies of life history variation is *Arabidopsis thaliana FLOWERING LOCUS C* (*FLC*). *FLC* determines the need for plants to overwinter and their ability to respond to prolonged cold in a process termed vernalization. This review highlights how molecular analysis of vernalization pathways has revealed important insight into antisense-mediated chromatin silencing mechanisms that regulate *FLC*. In turn, such insight has enabled molecular dissection of the diversity in vernalization across natural populations of *A. thaliana*. Changes in both cotranscriptional regulation and epigenetic silencing of *FLC* are caused by noncoding polymorphisms at *FLC*. The *FLC* locus is therefore providing important concepts for how noncoding transcription and chromatin regulation influence gene expression and how these mechanisms can vary to underpin adaptation in natural populations.

Contents

INTRODUCTION

A major goal of evolutionary biology is to understand the genotype changes that have enabled organisms to evolve new forms and functions and adapt to different environments. The era of "omics" promised major advances in this understanding. However, despite significant technological advancements, systems in which the mechanistic basis of evolutionary change has been defined at the molecular level remain rare. The study of life history variation in plants, specifically in the reference plant *Arabidopsis thaliana*, is one exception. Despite many hundreds of gene products functioning in the transition to flowering, a considerable proportion of life history variation has

been mapped to a small number of loci with large effects, likely because these loci have fewer pleiotropic effects than most.

An example of one of these loci is *FLOWERING LOCUS C* (*FLC*). Throughout the Brassicaceae, *FLC* is a major factor that determines the requirement for prolonged cold exposure to establish floral competency. This process, known as vernalization, optimally aligns flowering with the cessation of winter and the arrival of spring, thus maximizing reproductive fitness. Adaptation to the significant latitudinal range covered by *A. thaliana* (from near the equator to the Arctic Circle) has necessitated significant phenotypic change in the nature of the vernalization response across different accessions of *A. thaliana*. Regulation of *FLC* has been a major node through which this variation has evolved.

In this review, we first describe how analysis of the requirement for vernalization has led to detailed dissection of a mechanism for quantitative regulation of *FLC* transcription. This mechanism involves an RNA-mediated chromatin silencing mechanism, which is becoming an important paradigm in the general field of cotranscription, not only in plants. We then summarize our understanding of how winter is sensed and remembered through the cold-induced epigenetic silencing of *FLC*. The registration of the prolonged cold of winter provides an interesting example of how organisms extract and remember information from noisy environmental signals. Lastly, we discuss the important messages *FLC* has provided for evolutionary biology. The mechanistic analyses of vernalization in different *A. thaliana* accessions have defined how subtle changes in *FLC* regulation, mediated in some cases by its antisense transcripts, account for a large proportion of the natural variation in vernalization and flowering time. We place *FLC* into the group of iconic loci, such as the *Escherichia coli trp* operon (Yanofsky 1971, 1981) and the mammalian *beta-globin* locus (Grosveld et al. 1987, Myers et al. 1986), for which in-depth understanding gained from their mechanistic dissection has informed gene regulation generally.

The Decision to Flower and the Cues Regulating It

The timing of flowering signifies one of the most important developmental transitions during the life history of a plant, due to the significant influence of such timing on reproductive success. As a result, competency to flower is tightly controlled by a variety of cues, both endogenously generated and derived from the external environment (Simpson & Dean 2002). The complex regulatory network underpinning this control enables flowering time to be aligned with the environmental conditions that maximize fitness (Wellmer & Riechmann 2010). Such alignment is under strong selection, with drought escape capable of driving evolution of flowering time in only a few generations (Franks et al. 2007).

Plants sense their external environments constantly, perceiving and transducing many signals, ranging from light quality to temperature and nutrient status, into molecular outputs. Integration of these environmental signals produces stimulus-controlled, environmentally aligned flowering (Parcy 2005). The distinct physiological characteristics of different species were originally thought to be underpinned by a multitude of molecular pathways. The reality, however, appears to be simpler, with independent environmental or developmental cues converging to regulate a common set of downstream targets through a highly integrated network of pathways (Simpson & Dean 2002). The diversity in physiology and flowering in plant species can result from quantitative changes to the different inputs into the pathways of this integrated network (**Figure 1**). This framework provides a means of understanding the evolution of different reproductive strategies, as we describe below.

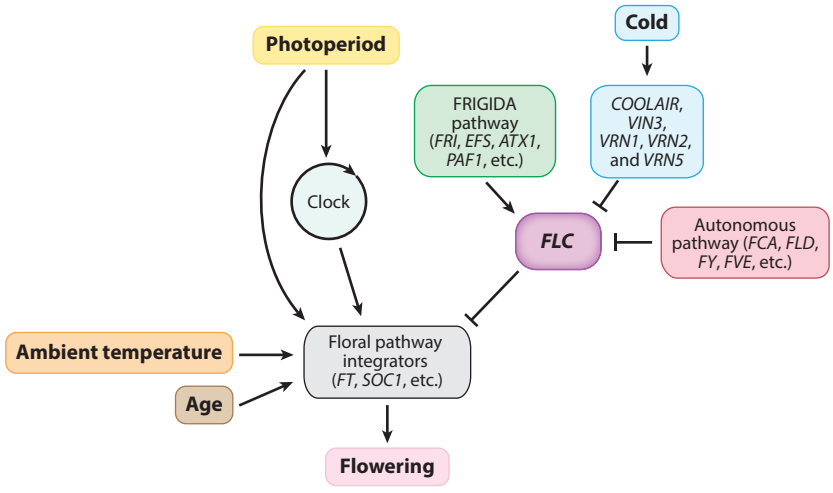


Figure 1

A variety of environmental and developmental cues independently regulate a set of floral pathway integrator genes to regulate the transition to flowering. Genetic analysis in *Arabidopsis thaliana* has defined a number of pathways that converge to regulate floral pathway integrator genes, which control the transition to flowering.

The Centrality of *FLC* in Flowering Time Regulation and Vernalization

Temperature can influence flowering in a variety of different ways; the prolonged cold of vernalization accelerates flowering (Chouard 1960), and heat can either hasten or suppress flowering, depending on the circumstances and context (Bouché et al. 2015). In many dicots, the prolonged cold response is mediated by a set of MADS-box transcriptional regulators (Alexandre & Hennig 2008), chiefly through *FLC* (Sheldon et al. 2000). *FLC* encodes a MADS-box transcription factor that prevents flowering (Michaels 1999) through repression of a set of floral activators, including *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF CO* (*SOC1*) (Lee et al. 2000). Because of this repressive effect, *FLC* expression must normally be reduced before *FT* can be efficiently activated and flowering induced (Searle et al. 2006). Other temperature stresses may also operate through *FLC*: Short-term cold stress induces *FLC* expression (Jung et al. 2013), and elevated ambient temperatures repress *FLC* expression (Gan et al. 2014, Sheldon 1999). Fluctuating temperatures have a complex output at *FLC*: The same mean temperature delivered as constant or alternating temperature profiles results in different consequences (Burghardt et al. 2016).

Conserved Hub, Diverse Responses: The Role of *FLC* in Generation of Variation

FLC has a conserved role in integrating temperature responses across multiple different plant lineages (Reeves et al. 2007). However, extensive variation exists both between and within different species in their requirement for vernalization. Within *A. thaliana* accessions, there is substantial diversity in whether cold exposure is necessary to establish competency to flower (Nordborg & Bergelson 1999). Commonly used lab strains lack a vernalization requirement and flower without prior cold exposure (Johanson 2000), but in contrast, many natural isolates flower very late in growth chamber conditions, unless first vernalized (Lee et al. 1993). Of those accessions that possess a vernalization requirement, diversity is also seen in the duration of cold required to fully accelerate flowering. Whereas some accessions fully vernalize after only 4 weeks, many others

require far longer (8 weeks or more) to completely suppress *FLC* expression and to establish competency to flower (Lempe et al. 2005, Shindo et al. 2005). This diversity is the result of variation at only a small number of loci, including *FLC* (Caicedo et al. 2004, Strange et al. 2011). This variation is in contrast to variation in pathways integrating other environmental cues, which is thought to be mediated by allelic effects at multiple loci (Lempe et al. 2005).

BALANCING ACT: ANTAGONISTIC INPUTS AT *FLC* DEFINE PREVERNALIZATION LEVELS OF EXPRESSION

The requirement for vernalization defines the reproductive strategy of a plant and is a product of *FLC* expression. By extension, the initial level of *FLC* expression prior to cold exposure is a significant determinant of the length of cold necessary to establish flowering competency. This expression represents the integrated output of two antagonistic pathways that set *FLC* expression: (*a*) FRIGIDA (FRI) and associated regulators that activate *FLC* expression and (*b*) the autonomous pathway (Koornneef et al. 1998), which represses *FLC* expression. Studies of these respective pathways not only have elucidated details of *FLC* regulation, but also have provided an understanding of cotranscriptional processes.

Don't Be FRIghtened: FRIGIDA and Activators of *FLC* Expression

FRI was initially identified during early genetic analyses of flowering time variation (Napp-Zinn 1957), which established that the vernalization requirement could be scored as a monogenic trait, despite the presence of many genetic modifiers. First characterized genetically in the 1950s (Napp-Zinn 1957), *FRI* was cloned in 2000 and was shown to encode a protein with coiled coil domains (Johanson 2000). Its mode of function remains unclear to date, although it seems to directly activate *FLC* transcription by interacting with the nuclear cap–binding complex, increasing the proportion of *FLC* mRNA that possesses a 5′ cap (Geraldo et al. 2009). Additionally, through interactions with FRL1, FES1, SUF4, and FLX, which together form a complex termed FRI-C, FRI facilitates recruitment of chromatin modifying factors to *FLC*, such as the chromatin remodeling SWR1 complex (delivering H2A.Z) and the histone methyltransferases EFS (a homolog of SET2 that catalyzes H3K36me3) (Choi et al. 2011), ATX1 (which catalyzes H3K4me3) (Pien et al. 2008), and SDG25 (Berr et al. 2009). The result is increased levels of chromatin modifications associated with high levels of transcription, such as H3K36 trimethylation, H3K4 trimethylation, and H3 and H4 acetylation (Hon et al. 2009). FRI thus produces a chromatin environment conductive to high levels of transcription, as well as directly affecting cotranscriptional processing of *FLC*.

Other, more general transcriptional activators are required to upregulate *FLC*. These include the RNA polymerase–associated factor 1 complex (Paf1C) (He et al. 2004), which regulates the transition into the elongation phase of transcription; RAD6-BRE1 (reviewed in Crevillén & Dean 2011), which is important for histone H2B ubiquitination; and SKIP, which is a component of the spliceosome that interacts with Paf1 (Cao et al. 2015). These activators support high levels of transcription in young seedlings but are also important in reactivating *FLC* expression in the embryo in vernalized plants; such reactivation is essential to ensure that seedlings do not "remember" vernalization of their parents (Yun et al. 2011).

Does It Make Sense? The Autonomous Pathway and Antisense Transcription

FRI activity on *FLC* expression is antagonized by a group of proteins that make up what has become known as the autonomous pathway because their activity appears to be largely independent of the environment (Koornneef et al. 1998). Together, the components of this pathway—FCA, FPA,

FY, FLOWERING LOCUS D (FLD), FLK, LUMINIDEPENDENS, and FVE—suppress expression of *FLC*, independently of FRI activity (Simpson & Dean 2002). The RNA processing and chromatin activities of this pathway form an RNA-mediated chromatin silencing mechanism that cotranscriptionally regulates *FLC* expression. This mechanism shows conceptual parallels with RNAi-mediated chromatin silencing in *Schizosaccharomyces pombe*. Three of the components—FCA, FPA, and FY—function as RNA-processing factors. These components are required for the processing of a collection of noncoding *FLC* antisense transcripts, collectively named *COOLAIR* (Hornyik et al. 2010, Liu et al. 2010). *COOLAIR* was one of the first long noncoding RNAs (lncRNAs) to be discovered with a demonstrated biological function. Alternatively spliced and polyadenylated, its transcripts fall into two broad classes: (*a*) one set that uses a proximal splice site along with several polyadenylation sites located within intron 6 of *FLC* and (*b*) a second set that uses a distal splice site and distal splice polyadenylation sites situated in the *FLC* promoter. FCA and FPA function partially redundantly to control 3′ processing and alternative splicing in *A. thaliana* (Hornyik et al. 2010, Liu et al. 2010) and—together with FY, CstF64, and CstF77 (3′ processing factors)—promote utilization of the proximal polyA sites. This activity results in a change in chromatin modification at the *FLC* locus via an FLD-dependent reduction in H3K4me2 levels, which suppresses *FLC* expression (Liu et al. 2010). The induced chromatin state leads to inefficient transcriptional initiation and slow transcriptional elongation, the latter of which is a feature first predicted through quantitative modeling and subsequently confirmed through experimental measurement of RNA polymerase elongation rates (Wu et al. 2016). It is thought that feedback from the chromatin state then reinforces choice of both the proximal splice and polyA sites, possibly through a kinetic coupling mechanism whereby coordination of polymerase elongation and transcript processing allows for changes in elongation rates to influence processing (Aebi et al. 1986, Bentley 2014, Marquardt et al. 2014). This mechanism involves a slow RNA polymerase II (PolII) elongation rate, a phenomenon well established in existing literature for other systems (de la Mata et al. 2003). In addition to these *COOLAIR*-dependent aspects of the autonomous pathway, FVE is thought to directly antagonize the action of FRI-C through its involvement in histone deacetylation (Ausín et al. 2004), specifically as a component of a HDA6-related complex (Gu et al. 2011).

The overall effect of this pathway is to coordinately and quantitatively influence both *FLC* and *COOLAIR* expression. Fluorescent in situ hybridization (FISH) analyses with the ability to detect single RNA molecules have clearly shown that *FLC* expression and *COOLAIR* expression are mutually exclusive at each locus (Rosa et al. 2016). This apparent contradiction is likely due to mutually exclusive transcription of the sense and antisense strands, both of which are similarly influenced by *trans* factors and the local chromatin environment. That sense-antisense transcription is mutually exclusive is another lesson from *FLC* that may be generally relevant.

Studies of *COOLAIR* have also yielded insight into the mechanisms underpinning the regulation of antisense transcription. The *COOLAIR* promoter forms an R-loop, an RNA-DNA hybrid structure formed through RNA invasion of the double-stranded DNA, which attenuates *COOLAIR* transcription (Sun et al. 2013). R-loops are frequently associated with genome damage but, at *COOLAIR*, are formed from nascent *COOLAIR* transcripts locally invading the DNA, preventing further antisense transcription. The homeodomain protein NDX1 stabilizes the R-loop, conferring a novel form of transcriptional regulation that is likely to play important roles in many genomes (Sun et al. 2013). Intriguingly, the *COOLAIR* promoter region is the target of small RNA–mediated chromatin silencing, with both 30-nt and 24-nt siRNAs (the latter associated with conventional RNA silencing pathways) produced by the region (Swiezewski et al. 2007). This is a relatively rare example of nonrepetitive DNA being the target of RNAi machinery delivering functionally important chromatin modifications (Swiezewski et al. 2007).

This complex network of antagonistic interactions thus sets the expression level of *FLC* prior to cold exposure, much like a dimmer switch allows for precise adjustment of light levels. Thus, despite *FLC* being primarily considered to be a switch gene whose on or off state determines competence to flower, *FLC* expression is subject to significant and meticulous quantitative regulation. As these initial levels determine the length of cold required to fully accelerate flowering, this quantitative control mechanism has important implications with regard to aligning flowering with environmentally optimal conditions.

WINTER IS COMING: VERNALIZATION AND THE USE OF EPIGENETIC REGULATION IN COLD REGISTRATION AND MEMORY

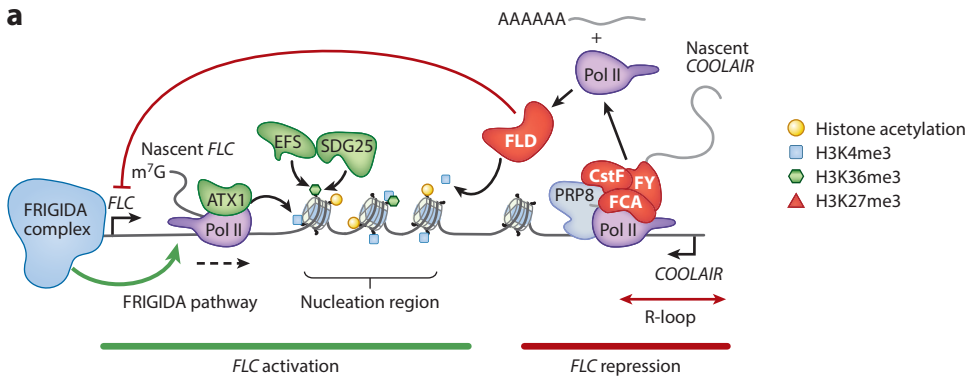
The process of vernalization involves the epigenetic silencing of *FLC* in response to cold exposure, the result of which is establishment of competency to flower. There are different temporal phases of vernalization (**Figure 2**). First, upon exposure to vernalizing cold, there is an initial shutdown of *FLC* transcription, which occurs during the initial few weeks and coincides with increased *COOLAIR* expression (**Figure 2*b***). Next, upon exposure to prolonged periods of cold, epigenetic silencing is mediated through the localized deposition of repressive chromatin modifications to a specific region within the *FLC* gene body (**Figure 2*c***). Finally, the silencing is stabilized through the spreading of these repressive marks across the entirety of the locus upon the return to warmth (**Figure 2*d***).

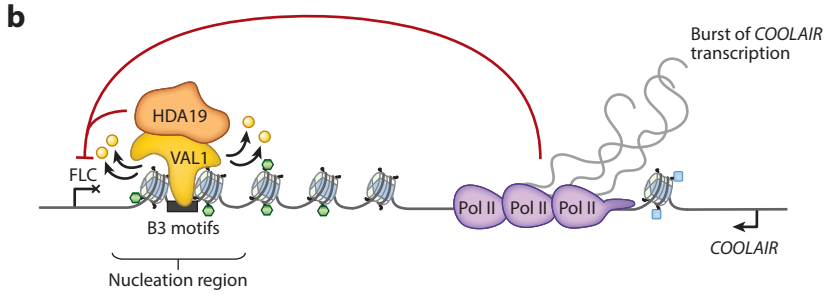
A Breath of (*COOL*)*AIR*: Initial Shutdown and the Complexities of Sense-Antisense Transcriptional Circuitries

The initial transcriptional downregulation of *FLC* expression that occurs within the first 2 weeks of vernalizing cold is mediated by the combinatorial action of several pathways. Upon cold exposure, the protein VAL1 that associates with two B3 *cis* motifs found in a specific region of *FLC* intron 1 (termed the nucleation region; see below) recruits the histone deacetylase HDA19, leading to reduced transcription (Qüesta et al. 2016). *COOLAIR* expression is also significantly upregulated and contributes to *FLC* transcriptional shutdown (Csorba et al. 2014, Rosa et al. 2016). *COOLAIR* expression reaches maximum levels after approximately 3 weeks of cold, coincident with disruption of a gene loop at *FLC* (Crevillén et al. 2013, Swiezewski et al. 2009; see also below). *COOLAIR* transcripts do not appear to be absolutely required for vernalization: Plants with T-DNA insertions that perturb *COOLAIR* expression do vernalize (Helliwell et al. 2011), but analysis of transgenic lines abrogated in *COOLAIR* production showed disruption of the coordination in a chromatin switch at the nucleation site, in addition to slower transcriptional downregulation of *FLC* (Csorba et al. 2014).

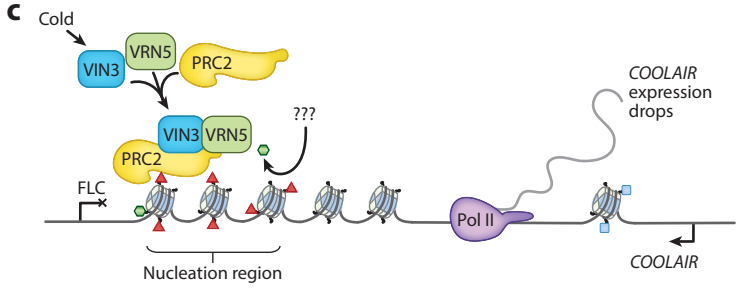
Tipping the Balance: Coordinated Shifts in Histone Modifications at *FLC* Underpin Epigenetic Silencing of the Loci

This initial transcriptional shutdown is followed by long-term epigenetic silencing of *FLC*, a process involving a series of coordinated chromatin changes at the locus. Prior to vernalization, *FLC* chromatin is enriched in a number of histone modifications associated with actively transcribed genes (Li et al. 2007), including H3K4me3, ubiquitination of histone 2B, histone acetylation, and (perhaps most notably) H3K36me3. Upon cold exposure, these marks are dynamically removed from the locus and are replaced with H3K27me3, a modification associated with Polycomb-repressed genes (Margueron & Reinberg 2011, Young et al. 2011). In particular, H3K27me3

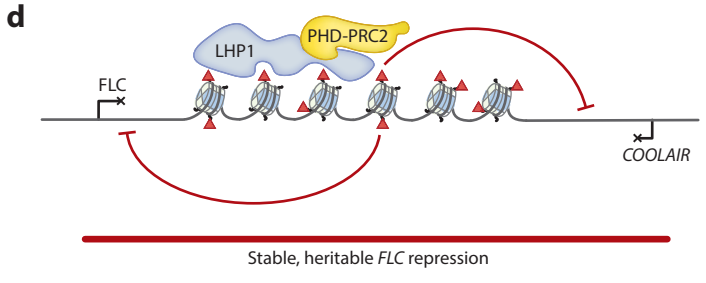




COOLAIR induction and histone deacetylation at the nucleation region



Deposition of H3K27me3 at the nucleation region



and H3K36me3 show opposing profiles (Yang et al. 2014), with this antagonism thought to be functionally important in the establishment of mutually exclusive chromatin states, a hypothesis generated through mathematical modeling of histone dynamics at *FLC* (Angel et al. 2011). This modeling also predicted a physical association between antagonistic histone modifiers, specifically of the H3K36me3 methyltransferase SDG8 and the H3K27me3 demethylase ELF6; such a prediction was recently confirmed experimentally (Yang et al. 2016). Additionally, such modeling highlights the importance of both the functional antagonism of histone modifications and the coupling of activities regulating these antagonistic states, a feature that is likely to be common to chromatin-based transcriptional regulation.

Initially, H3K27me3 deposition is limited to a specific section of the *FLC* gene termed the nucleation region. This region spans approximately three nucleosomes and is downstream of the transcriptional start site, centered over exon 1 and the start of intron 1 (Finnegan & Dennis 2007, De Lucia et al. 2008). This localized accumulation of H3K27me3 is mediated by the PRC2 complex, composed of members of the Polycomb protein family—VRN2, SWINGER, FIE, and MSI1—and a set of PHD proteins. One PHD finger protein, VIN3, is cold induced (Sung & Amasino 2004), whereas the other, VRN5, is constitutively expressed. VIN3 and VRN5 are thought to increase PRC2 histone methyltransferase activity, resulting in increased accumulation of H3K27me3 (Greb et al. 2007, Wood et al. 2006).

What recruits PRC2 to specific loci has been a matter of some debate in the literature. Mutational analysis of *FLC* has informed this debate; a single mutation within the noncoding nucleation region blocks binding by VAL1 (Qüesta et al. 2016), the sequence-specific factor responsible for recruiting PHD-PRC2 to the nucleation region. VAL1 also interacts with components of the conserved apoptosis- and splicing-associated protein complex, which interact with PHD proteins VIN3 and VRN5, aiding PRC2 recruitment. Recruitment of PHD-PRC2 to the nucleation region has also been proposed to involve another lncRNA, *COLDAIR* (Heo & Sung 2011), with recruitment facilitated through an interaction with the histone methyltransferase subunit of PRC2, CLF. However, the function of this lncRNA remains enigmatic and, unlike *COOLAIR*, has yet to be observed in other members of the Brassicaceae (Castaings et al. 2014).

Frozen in Time: Memory of the Cold and Stable Maintenance of Epigenetic Silencing

The cold-induced nucleation of Polycomb silencing induces transcriptional repression, which is then followed by the establishment of stable, heritable silencing (through the spread of both Polycomb silencing and H3K27me3 across the entirety of the locus) upon the return to warmth. *FLC* thus represents a rare system in which establishment and maintenance of Polycomb silencing

Figure 2

FLC regulation before, during, and after the different stages of vernalization. (*a*) Setting *FLC* expression levels before cold exposure. Antagonistic pathways activating (*green*) and repressing (*red*) *FLC* expression are shown. *FLC* chromatin is schematically illustrated, with *FLC* and *COOLAIR* transcription start sites shown. (*b*) Transcriptional shutdown by cold exposure. A burst of *COOLAIR* transcription facilitates *FLC* transcriptional shutdown in the cold, together with VAL1 recruitment of HDA19 to the nucleation region. (*c*) Nucleation of silencing during cold. Association of the PHD proteins VIN3 (cold induced) and VRN5 with the PRC2 complex results in the deposition of H3K27me3 at the nucleation region within *FLC*, leading to Polycomb-mediated silencing. (*d*) Epigenetic maintenance of silencing. Upon the return to warmth, H3K27m3 spreads across the entire locus. This spreading, along with association of LHP1, establishes heritable silencing that is maintained across many cell divisions.

are both spatially and temporally separated, a feature that allows one to easily dissect the sequence of events occurring. Indeed, a striking conclusion made possible by these distinct dynamics was that quantitative changes in epigenetic silencing are the result of digital switching in an increasing proportion of cells, and not quantitative changes at individual loci (Angel et al. 2011, Berry et al. 2015). This conclusion was predicted by quantitative modeling (and subsequently confirmed with experimental work) and was based on a relatively simple concept: Chromatin states are bistable, and there is dynamic switching between states. The modeling predicted that H3K27me3 deposition at a single locus occurs in a binary on/off or digital manner (Angel et al. 2011); such digital registration of cold helps in the integration of the noisy, interrupted temperature signals that are commonplace in real-life environments (Angel et al. 2015).

One question that remains, however, is why H3K27me3 spreading and epigenetic switching to a stably silenced state occur only upon the return to warmth. There is the possibility that the process of spreading itself requires DNA replication (Finnegan & Dennis 2007): The catalytic subunit of *Arabidopsis* DNA polymerase α is required for stable maintenance of histone modifications at *FLC*, with mutants exhibiting unstable maintenance of H3K27me3, resulting in reactivation upon the return to warmth (Hyun et al. 2013). More recently, DNA polymerase ε (Polε) was shown to recruit components of PRC2 to mediate epigenetic silencing of genes; the catalytic subunit of Polε, ESD7, interacts with CLF, EMF2, and MSI1 to facilitate PRC2 recruitment to the chromatin of, in this case, *FT* and *SOC1* (Del Olmo et al. 2016). DNA replication may therefore play an active role in propagating the repressive chromatin marks across the entirety of the *FLC* locus from their initial focal point in the nucleation region (Zaratiegui et al. 2011). Regardless, upon the return to warmth, the spread of Polycomb silencing and concomitantly of H3K27me3 across the gene body (Finnegan & Dennis 2007, De Lucia et al. 2008) establishes a large enough patch of H3K27me3 to maintain Polycomb silencing. Analysis of two *FLC* fluorescent protein fusions in the same cell has showed that the local chromatin environment is sufficient to instruct epigenetic inheritance of the silenced state (Berry et al. 2015), highlighting and confirming that the spread of H3K27me3 across the gene body facilitates the maintenance of Polycomb silencing and building on previous work proposing that maintenance is achieved through a histone-based positive feedback mechanism (Angel et al. 2011).

This chromatin-instructed maintenance of silencing through cell divisions is in turn dependent on a number of protein factors, including the *LHP1* locus (Mylne et al. 2006, Sung et al. 2006), which physically associates with the *FLC* locus (Sung et al. 2006). Able to bind H3K27me3 (Turck et al. 2007), LHP1 forms a complex with EMF1 and a histone H3K4 demethylase that plays a role in maintenance of the epigenetically silenced state at *FLC*, but also at *FT* (Wang et al. 2014).

However, in plants, the germ line arises from somatic tissues, necessitating (*a*) the existence of a mechanism for erasure of epigenetic modifications accrued at *FLC* during a plant's lifetime and (*b*) a reset of expression to prevent inheritance of the vernalized state. The jumonji domain–containing protein ELF6, which possesses H3K27me3 demethylase activity, contributes to the resetting of the vernalized state in the plant embryo (Crevillén et al. 2014).

Let's Get Physical: *FLC* Epigenetic Silencing Is Accompanied by Significant Structural Changes

An emerging theme in gene regulation is the importance of physical position and spatial situation within the nucleus (Lanzuolo et al. 2007, Maison et al. 2002). A similar story has been found at *FLC*, at which, in addition to the dynamic transcriptional and epigenetic reprogramming, significant changes to nuclear organization and physical structure occur during vernalization. Chromosome conformation capture analysis has revealed the presence of a gene loop at *FLC*, involving a physical

LESSONS FROM *FLC*

- Functionality and importance of switching between mutually antagonistic chromatin states
- Temporal separation of nucleation and spreading for stable inheritance
- Local chromatin environment sufficient for mitotic inheritance of Polycomb silencing
- Resetting via H3K27me3 demethylation preventing transgenerational inheritance
- Coordination of sense-antisense promoter function by gene loops
- Clustering of alleles during epigenetic switching
- Chromatin state coordination of transcriptional firing and elongation
- R-loop stabilization suppressing transcription
- Mutually exclusive sense-antisense transcription

interaction between the flanking 5′ and 3′ regions. Intriguingly, although the loop is unaffected by mutations to various components involved in establishing expression levels of the locus, it is significantly disrupted upon cold exposure during vernalization. Thus, the loop may play a role in early stages of transcriptional shutdown, prior to the switch to a silenced chromatin state (Crevillén et al. 2013).

Additionally, live-cell imaging of *FLC-lacO* revealed that *FLC* alleles in mitotically dividing cells physically cluster together during cold and remain so upon the return to warmth. This clustering is dependent on the above mentioned PRC2 components that are involved in the establishment of the *FLC* silenced state, but not on LHP1, which helps maintain silencing (Rosa et al. 2013). This finding lends support to the suggestion that physical clustering in the nucleus both represents a physical manifestation of Polycomb nucleation and is likely to be a conserved and important aspect of gene regulation. Vernalization pathways have thus revealed important insights into many aspects of chromatin silencing mechanisms (see sidebar entitled Lessons from *FLC*).

FROM DIFFERENCES, CONSERVED PRINCIPLES: *FLC* AND THE MOLECULAR MECHANISMS UNDERPINNING ADAPTATION

Although *FLC* is present in many dicots, in which it has a central role in vernalization, significant diversity in the vernalization response exists throughout the plant kingdom. First, there is variation between species (as well as between accessions of a single species) as to whether they possess a vernalization requirement. Second, for those that require vernalization, there is also extensive variation in the length of cold required. Finally, there is significant variation in life history structure and in how the vernalizing requirement is thus accommodated: Whereas many species adopt annual flowering habits, others are perennial and live for many years, flowering multiple times during that period.

Same Species, Different Stories: Coding Variation in Key Vernalization Components Underpins Distinct Life History Strategies

Across *A. thaliana*, there is significant diversity in the reproductive strategies utilized (**Figure 3**) (Brachi et al. 2010, Lempe et al. 2005, Shindo et al. 2005, Stinchcombe et al. 2004). The genetic basis for these differences occurs through a range of loci, but predominantly through allelic variation at *FRI* and *FLC*. Many rapid cyclers (accessions lacking a vernalization requirement) contain mutant alleles of *FRI* that lead to a loss of functionality, including both nonsense mutations as well as alterations leading to premature termination. Such a phenomenon appears to have evolved

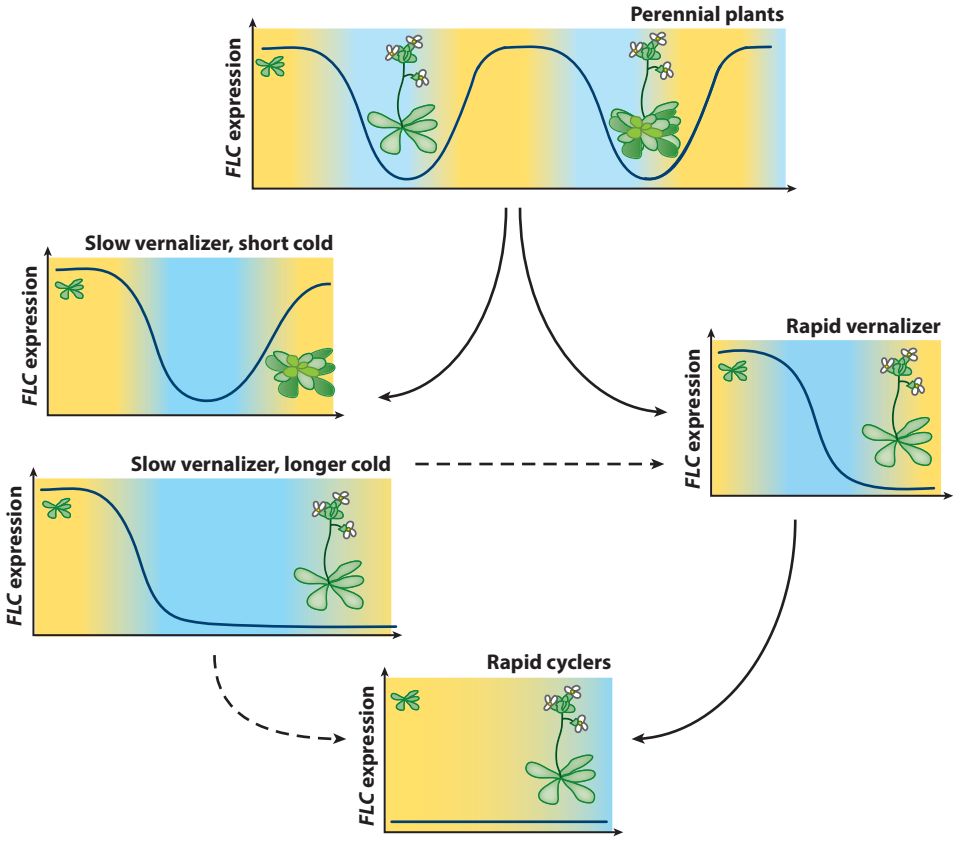


Figure 3

Illustration of different reproductive strategies and the putative evolutionary progression between them. In perennial plants, cold-induced *FLC* repression is not stable, with reactivation following the return to warmth facilitating the perennial habit. In slowly vernalizing winter annuals, exposure to short periods of cold similarly results in incomplete silencing and reactivation of *FLC* expression upon the return to warmth, with stable epigenetic silencing occurring only after longer cold exposure. In rapidly vernalizing winter annuals, epigenetic silencing of *FLC* requires only short cold exposure. Rapid cyclers lack a vernalization requirement entirely; this strategy is usually the result of mutations to the *trans* factor FRI, *FLC*, or both.

independently at least 20 times across *A. thaliana* populations (Johanson 2000, Shindo et al. 2005), highlighting divergent evolutionary routes to convergent phenotypes. Weak *FLC* alleles also cause a rapid cycling habit (Michaels et al. 2003), with Van-0 in particular identified as possessing an *FLC* nonsense mutation that confers early flowering (Werner et al. 2005). By contrast, accessions with a strong vernalization requirement generally possess active copies of both *FRI* and *FLC*; *FRI*'s activating effect increases expression of *FLC*, leading to the requirement for vernalization.

A Gentler Touch: Finer-Scale Modulations to Vernalization Responses Across the Accessions

Although significant disruption to either *FRI* or *FLC* can be sufficient to provoke the switch from an annual-based life history to a rapidly cycling one, variation at these loci (in particular

FLC) also has the capacity for finer modulation of vernalization, allowing for adaptation to local climates (Ågren et al. 2013, Grillo et al. 2013, Shindo et al. 2006, Strange et al. 2011). Analysis of functional diversity at *FLC* across 1,307 *A. thaliana* accessions revealed the existence of multiple functionally distinct *FLC* haplotypes, each defined by specific noncoding variation (with almost complete coding sequence conservation) (Li et al. 2014). A total of 20 haplotypes were identified, 5 of which predominate worldwide. Analysis of accessions belonging to these 5 revealed that the distinctive vernalization phenotypes are a result of altered *FLC* silencing kinetics (Li et al. 2014).

Lövvik-1 and Var2–6 *FLC*s: Divergent Routes to Convergent Phenotypes

Mechanistic studies of individual alleles have greatly informed our knowledge of the function of nucleotide sequence diversity at *FLC* in generating distinct vernalization responses. One notable example is the *FLC* allele of Lövvik-1 (Lov-1), an accession from the High Coast region of Northern Sweden, near the northern limit of the *Arabidopsis* range. It is one of the most slowly vernalizing accessions yet studied (Shindo et al. 2006), requiring 3 months of vernalizing cold at 5°C to fully accelerate flowering. The slow rate of vernalization contrasts with accessions like Edi-0, in which the vernalization response is fully saturated after only 4 weeks of cold.

Analysis of the genetic basis of the slowly vernalizing phenotype in Lov-1 identified a region near the 5′ end of *FLC* containing 4 noncoding single-nucleotide polymorphisms (SNPs) responsible for the distinct vernalization response (Coustham et al. 2012). This genetic variation quantitatively modulates PRC2-mediated epigenetic silencing and increases the time required to reach the necessary H3K27me3 levels (Coustham et al. 2012). Exactly how these SNPs influence the mechanism remains to be determined. Lov-1 also possesses an altered optimum vernalization temperature: Whereas most accessions vernalize equally efficiently at 2°C, 5°C, and 8°C, Lov-1 preferentially vernalizes at 8°C, a phenomenon that results in vernalization occurring during autumn (Duncan et al. 2015). This seasonal shift in the timing of vernalization likely represents an adaptation to the extreme winters of Northern Sweden, facilitating rapid flowering upon snowmelt.

In contrast to the altered chromatin dynamics identified in Lov-1, the slow vernalization response observed in Var2–6, another Swedish accession from a different haplotype group, is due to altered *COOLAIR* splicing, specifically splicing of the distally polyadenylated *COOLAIR*s, relative to other accessions (Li et al. 2015). Further molecular characterization revealed that a single noncoding nucleotide change in intron 1 of *FLC*, specifically a change from a T to a G, was sufficient to alter splice site utilization such that the last distal exon was truncated by 54 bp and an additional 64 bp exon was incorporated. This altered splicing of the *COOLAIR* antisense transcript significantly increases *FLC* transcription, necessitating longer cold for Var2–6 to fully vernalize. Molecular characterization of transgenic lines abrogated in *COOLAIR* production showed that this effect is dependent on *COOLAIR*, with altered *COOLAIR* function increasing *FLC* expression via a cotranscriptional mechanism involving capping of the nascent *FLC* transcript (Li et al. 2015). Thus, across evolutionarily distinct accessions, different aspects of the vernalization mechanism appear to have been modulated to achieve functionally similar alterations to vernalization response. That the process of vernalization can apparently evolve so rapidly may account for the wide geographic range inhabited by *A. thaliana* accessions.

Playing It Cool: Vernalization Requirements and Life History Strategy

Beyond these intraspecific comparisons, examination of the mode of action of *FLC* in closely related perennial species such as *Arabis alpina* has contributed significantly to our understanding of the evolutionary events underpinning the production of these distinct life history strategies

(**Figure 3**). Whereas annual plants complete their entire life cycle within a single year, perennials live for many years and flower repeatedly. Studies of vernalization in *Arabidopsis halleri* and *A. alpina* revealed that the *FLC* homolog shows seasonal cycles of repression in response to cold exposure and that, as with *A. thaliana*, *FLC* suppression is responsible for initiating the onset of flowering (Aikawa et al. 2010, Wang et al. 2009). Unlike the case of *A. thaliana*, however, for *A. alpina* suppression is not stable and occurs only transiently. This alteration between suppressed and active *FLC* states allows a fraction of the meristems to remain vegetative while allowing others to flower in response to the cold environment. Further analysis of the differences between *A. thaliana* and *A. alpina* highlighted the conservation of H3K27me3 in generating a silenced *FLC* state, with reactivation occurring due to the loss of this histone mark (Wang et al. 2009). Differences in the regulation and maintenance of this chromatin modification therefore contribute to the life history variation seen between the two species and in turn raise the question of whether the diversity in vernalization requirements in winter annual *A. thaliana* accessions might represent derivations of an ancestral perennial condition. Small changes in *FLC cis*-regulatory sequences might affect the degree of epigenetic silencing, converting a perennial to a winter annual and thus allowing a different geographical range to be colonized (Hoffmann 2005).

OLD QUESTIONS, NEW PLAYERS: *FLC*, EVOLUTIONARY ADAPTATION, AND LONG NONCODING RNAS

Small Changes, Big Consequences: Noncoding SNPs as Drivers of Major Evolutionary Changes

That such small changes to noncoding regions of the *FLC* locus effect such large changes to the phenotype of *A. thaliana* informs the debate on the different types of molecular variation that underpin macroscopic adaptive evolution. The relative contributions of regulatory and protein-based changes have long been controversial (King & Wilson 1975), and although there is evidence for the role of each in adaptive evolution (Halligan et al. 2013, Jones et al. 2012), the relative predominance of protein coding (Kvitek & Sherlock 2011), noncoding (Jones et al. 2012), and *cis*-regulatory (Wray 2007) mutations has been strongly contested.

Studies of vernalization have identified a role for both *cis* noncoding variation and protein-based changes, but the phenotypic consequences are somewhat different. Loss-of-function mutations in *FRI* (Johanson 2000) and *FLC* (Michaels et al. 2003) allow plants to flower without need for vernalization and to adopt a rapid cycling habit, whereas *cis* noncoding variation has far more subtle effects, allowing for fine-tuning of the vernalization response to the specific climatic conditions of particular latitudes and different environments (Li et al. 2014, Scarcelli et al. 2007). In both cases, this variation has been the product of only a small number of alterations: either single nonsense mutations or a few SNPs in noncoding regions of *FLC*.

A New Look at the Previously Ignored: Noncoding Variation and Long Noncoding RNAs

Of particular interest is that variation at the *FLC* locus potentially offers insight into long noncoding transcript functionality (lncRNAs). lncRNAs are often poorly conserved in sequence (Ulitsky et al. 2011), and some investigators have argued that most lncRNAs simply represent transcriptional noise (van Bakel et al. 2010). However, mounting evidence suggests that many lncRNAs play a variety of roles in diverse processes (Mercer et al. 2009). This functionality appears to be

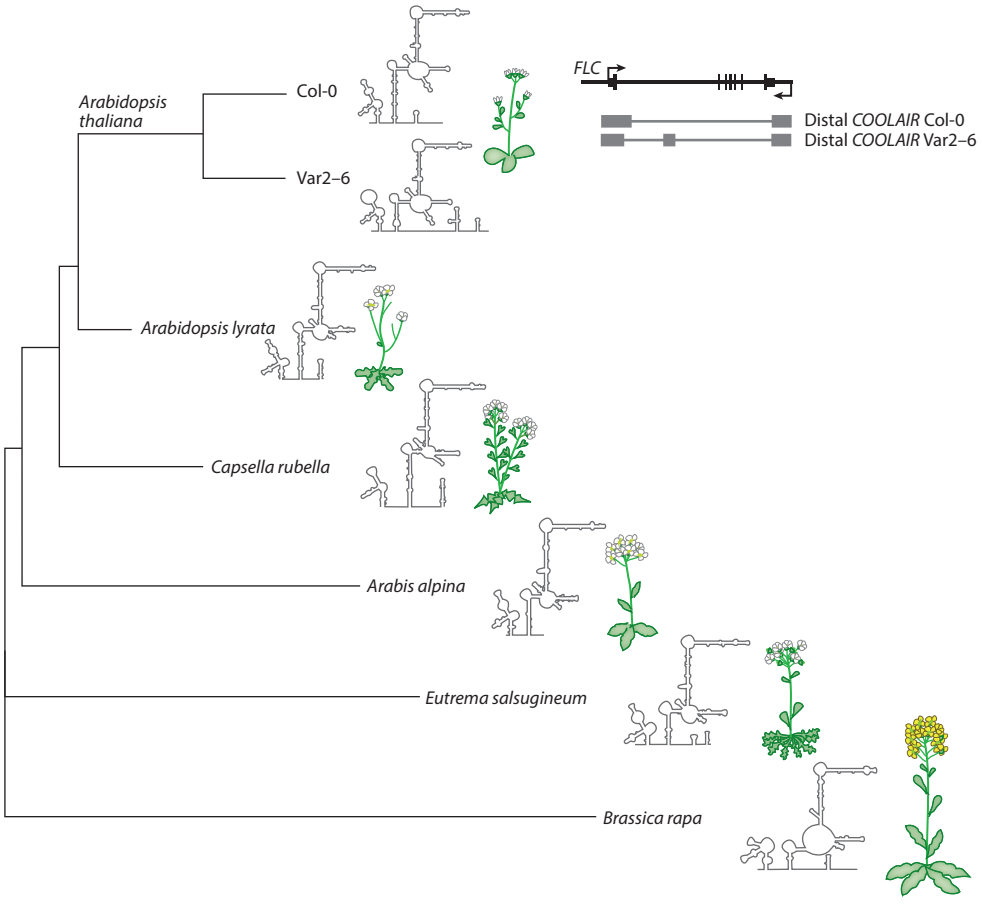


Figure 4

Variation in secondary structure of distal *COOLAIR* across the Brassicaceae. Distal *COOLAIR* variants possess a complex and intricate secondary structure, displaying significant evolutionary conservation despite low sequence similarity. Although there are similarities, there are also a number of structural differences, most notably the length of the helix preceding the first right-hand-turn motif. Such structural differences may have functional consequences, as supported by evidence from studies conducted in the slowly vernalizing accession Var2–6. In the instances presented here, secondary structures were either determined through in vitro chemical probing experiments or based on predictions from the sequences. Adapted from Hawkes et al. (2016).

primarily a result of secondary (and higher-order) RNA structures, rather than of exact sequence (Fang et al. 2015, Somarowthu et al. 2015, Zhang et al. 2010).

Recent determination of the in vitro secondary structure of spliced *COOLAIR* variants across a wide range of Brassicaceae species has revealed significant and elaborate secondary structures, despite the lack of *COOLAIR* primary sequence conservation (**Figure 4**) (Hawkes et al. 2016). The distally polyadenylated *COOLAIR* variants from *Arabidopsis lyrata*, *Capsella rubella*, and *Eutrema salsugineum* share significant secondary structure conservation with *A. thaliana*. However, there are differences, which are notably seen in comparisons between the rapidly vernalizing Col-0 *FRI-Sf2* and the slowly vernalizing Var2–6 accessions. These differences are the result of the altered *COOLAIR* splicing in Var2–6, the product of a single SNP adjacent to the distal splice site. This altered structure thus modulates *COOLAIR*'s functionality, eliciting the phenotypic

variation observed. Modulations in lncRNA secondary structure and function may therefore play an important role in adaptive evolution. Indeed, one can speculate that the freedom from protein coding capacity and higher natural expression variation across different cell types (Kornienko et al. 2016) may make lncRNAs particularly suited to rapid and dynamic reshaping by variation and evolutionary forces. Supporting this possibility is recent evidence of rapid evolutionary turnover of the *roX1* and *roX2* lncRNAs across a wide range of *Drosophila* species, despite broad conservation of the patterns of interactions of these lncRNAs with genomic DNA required for function (Quinn et al. 2016).

CONCLUSION: TIME TO COME IN FROM THE COLD?

By integrating information from several pathways to condition the transition to reproduction, *FLC* acts as a switch in the decision to flower in optimal environmental conditions. Such a central position within the vernalization gene regulatory network confers *FLC* with characteristics similar to those of genes acting as input/output devices that often underlie repeated evolution (Stern & Orgogozo 2008). Studies that examine both the regulatory mechanisms and population genetic patterns at such loci have the potential to provide important insights into the nature of adaptive polymorphisms and into how the regulatory mechanisms acting on these genes may constrain or provide flexibility in trait evolution (Losos 2011, Stern 2013).

Analysis of the mechanistic processes underpinning the vernalization requirement and response has given us an intricate picture of cotranscriptional regulation and epigenetic silencing at *FLC*. This understanding has, in turn, enabled molecular identification of the causes of the diversity in the nature and efficacy of the vernalization requirement and response across natural populations of *A. thaliana*. The surprise has been that subtle changes, involving SNPs or very few nucleotide polymorphisms, have enabled adaptation of the vernalization process to different environments. Therefore, such variation likely evolved readily in response to changing environmental pressures.

These conclusions have particular relevance, given the burgeoning of the field studying epigenetic variation in the context of adaptation and evolution. Although there are exceptions (e.g., Silveira et al. 2013), such studies for the most part lack a detailed description at the single-gene level. They instead overwhelmingly rely on statistical inferences from whole-genome analyses, focusing primarily on DNA methylation and analyzing variation induced independently of DNA sequence changes. The study of *FLC* has demonstrated the power of integrating information from a wide range of techniques and at a variety of different functional levels from the molecular to the macroscopic. In doing so, such investigation has illustrated the potential for altered epigenetic regulation, underpinned by genetic variation, to drive adaptation to new and changing environments. These insights should help guide future studies using whole-genome methods.

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