How Plants Feel The Cold: Dissecting Distinct Vernalisation Responses Across *Arabidopsis thaliana* Accessions.

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Abstract

The winter annual *Arabidopsis thaliana* requires a prolonged period of cold in order to establish competency to flower, in a process known as vernalisation. Accessions of *Arabidopsis* worldwide show distinct and highly variable vernalisation responses and a significant amount of this variation has been mapped to a single locus- FLOWERING LOCUS C (*FLC*). Whilst the source of the variation is known, the particular polymorphisms, and the exact nature by which they influence different aspects of the vernalisation process remains comparatively unexplored.

This thesis addresses two questions: firstly, what genetic features of *FLC* across different accessions contribute to their distinct vernalisation responses? And secondly, how does the process of vernalisation in different accessions differ to produce these distinct vernalisation requirements? The work presented here addresses these questions from a number of different perspectives.

Chromatin immunoprecipitation (ChIP) experiments point to the role of altered chromatin dynamics as the driver of different vernalisation requirements in two different *Arabidopsis thaliana* accessions. Expression analysis of transgenic chimeric *FLC* loci containing domains from both rapidly and slowly vernalising accessions highlights the importance of particular single nucleotide polymorphisms (SNPs) in affecting *FLC*'s propensity to reactivate upon return to warmth after a short period of cold. Finally, analysis of *FLC* suppression dynamics during cold exposure across a range of accessions illustrates mechanistic differences in vernalisation as the driver of distinct vernalisation requirements.

The work presented in this thesis examines the natural diversity in vernalisation requirements across *Arabidopsis thaliana* accessions in order to better resolve the nature of such variation, both in terms of its causative features as well as the underpinning mechanism. In doing so, it highlights that a small number of polymorphisms are able to alter the process of vernalisation in significant ways, and that, additionally, the way in which the vernalisation mechanism has been altered varies across accessions.

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Chapter 1- Introduction.

1.1 The Decision to Flower And Its Importance For Plant Reproductive Success.

The floral transition represents perhaps the most important developmental event during a plant's life history, signifying as it does, the mechanism by which genetic information is propagated to a new generation. As a result of this importance, this key developmental event is highly and meticulously regulated, with competency to flower tightly controlled by a diversity of different cues, both those originating from the external environment, as well endogenously generated signals (Figure 1)[1]. The result is a vast and complex regulatory network[2], which in turn gives precise and highly sensitive control of flowering time. This is essential in order to align flowering time with optimal environmental conditions, which in turn is necessary to maximise reproductive success. There is considerable evidence of this being strongly selected for, with natural selection for drought escape shown to be capable of driving adaptive evolution in only a few generations[3].

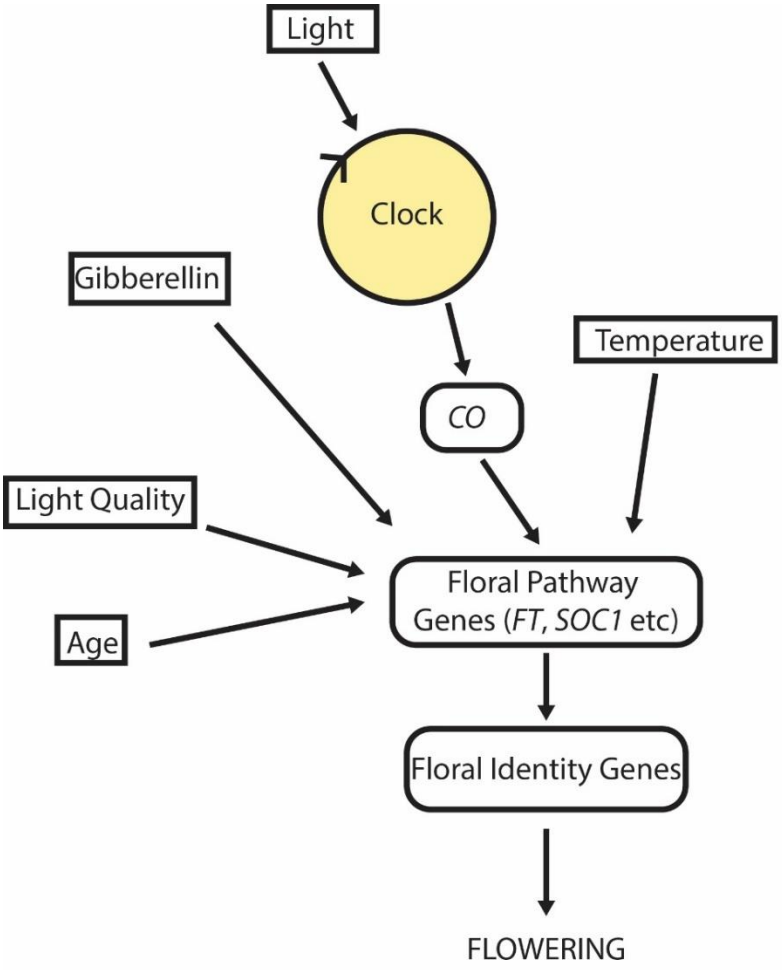

Figure 1: Schematic illustrating the types of endogenous and exogenous inputs that affect and modulate flowering competency and flowering time. The vast majority of inputs converge on so called "floral integrators" which in turn affect expression of the floral identity genes that control the floral transition.

Of the many environmental stimuli plants monitor[4], temperature is one of the best characterised and can influence flowering in a variety of ways: for example, whereas heat can either hasten or suppress flowering depending on the circumstances and context[5], prolonged periods of cold have been shown to accelerate flowering, in a process known as vernalisation[6]. The study and mechanism of the latter will form the basis of this thesis.

Of the diversity of temperature responses plants manifest, a large number are integrated at a single genetic locus, *FLC*[7]. *FLC* encodes a MADS-box transcription factor that acts as a repressor of flowering[8] through repression of the floral activators, *FT* and *SOC1*[9]. Given this repressive effect, its expression must be reduced before *FT* can be activated, and flowering induced[10]. Its initial expression is set by the antagonistic action of two pathways (Figure 2), the autonomous and FRIGIDA pathways- this is then modulated and altered by temperature in a variety of ways. Whereas short term cold stress induces *FLC* expression[11], elevated ambient temperatures and prolonged cold exposure (the process of vernalisation) both repress expression and thereby hasten flowering[12,13], the latter occurring through a series of coordinated chromatin changes that ensue following cold exposure[14] and epigenetically silence the gene. The specific chromatin modification involved in this process is trimethylation on lysine 27 of histone H3[15], which maintains repression of expression and concomitant establishment of competency to flower. The exact mechanism by which this is achieved will form the basis of the following sections.

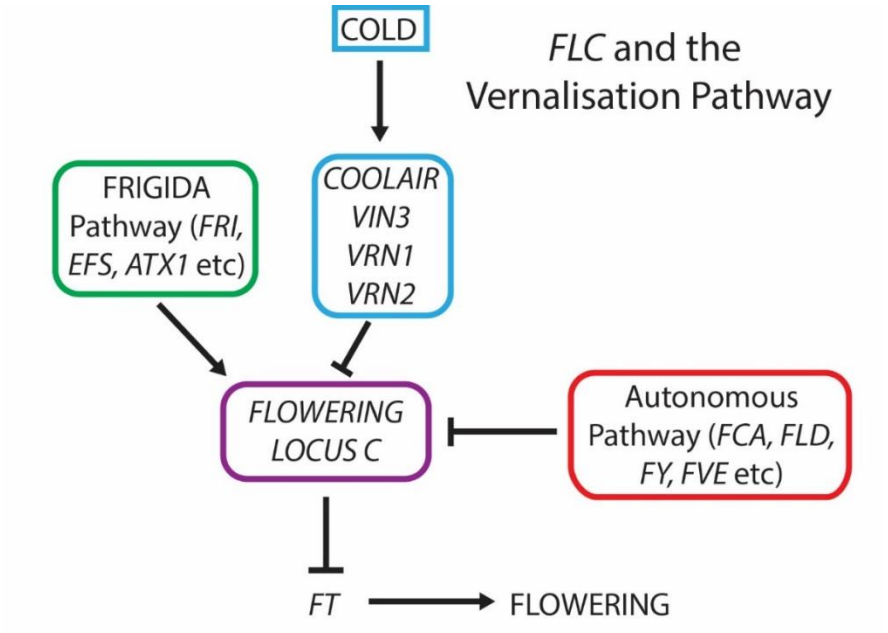

Figure 2: Illustration of the components and activites of the various pathways integrated by *FLOWERING LOCUS C* (*FLC*), including the autonomous and FRIGIDA pathways, the balance of which sets *FLC* expression prior to cold exposure, as well as components of the vernalisation pathway, which are induced in response to cold and act to suppress *FLC* expression.

1.2 An Introduction to Vernalisation and the Registration of Cold.

Vernalisation represents the coordinated epigenetic silencing of *FLC* in response to cold exposure, and with it, the cessation of *FT* repression and establishment of competency to flower. It can be delineated into discrete temporal phases according to the factors involved and their effect on *FLC* expression (Figure 3), the exact details of which will be discussed below.

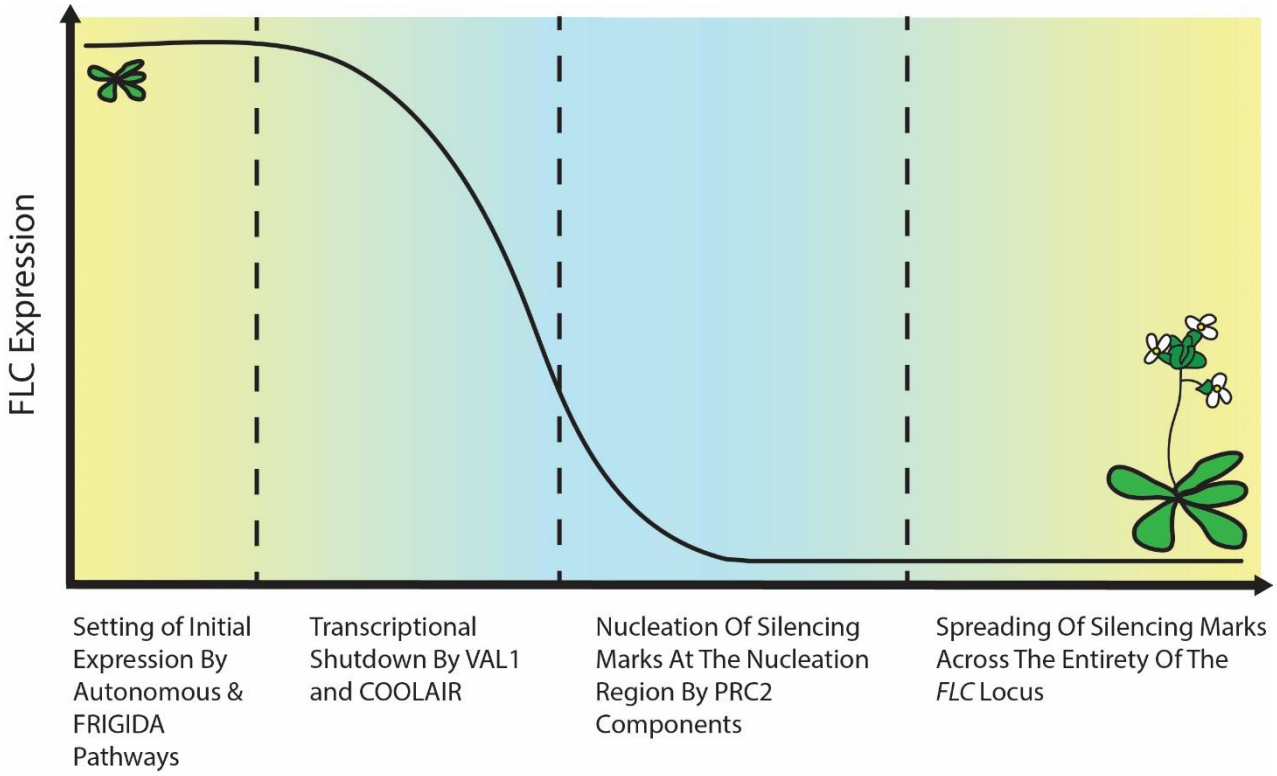

Figure 3: Illustrating the discrete temporal phases of the vernalisation process, the result of which is the mitotically stable epigenetic silencing of *FLC* expression. Initial levels of *FLC* are set by the coordinated and antagonistic activities of the autonomous and FRIGIDA pathways. Upon cold exposure, *FLC* transcription quickly decreases due to the effects of *VAL1* and *COOLAIR* induction. This transcriptional shutdown is followed by nucleation of the repressive silencing mark H3K27me3 at a small region spanning the transcriptional start site of *FLC*, exon 1 and part of intron 1, known as the nucleation region. Upon return to the warmth, this repressive epigenetic mark quickly spreads across the entirety of the *FLC* locus, resulting in silencing of expression that is stable across cell divisions.

1.3 Setting the Balance: Antagonistic Inputs At *FLC* Define Pre-Vernalisation Levels.

Levels of *FLC* prior to cold exposure are a product of the integrated output of two opposing, antagonistic pathways that together balance and maintain *FLC* expression prior to cold exposure (Figure 4). These are the FRIGIDA (FRI) pathway, responsible for activating *FLC* expression and the autonomous pathway[16] which acts to repress expression. FRIGIDA acts to increase the levels of *FLC* transcripts[17]- it interacts with the nuclear cap-binding complex, increasing the proportion of *FLC* mRNA that possess a 5' cap[18], as well as recruiting a variety of chromatin modifying factors to the locus, such as the chromatin remodelling SWR1 complex and the histone methyltransferases EFS[19], ATX1[20] and

SDG25[21]. The result is increased levels of chromatin modifications associated with high levels of transcription, such as trimethylation of lysine 36 and 4 on histone 3 (H3K36me3 and H3K4me3 respectively), as well as acetylation of histones 3 and 4.

Antagonising the efforts of the FRI pathway is the "autonomous pathway", which acts to suppress expression of *FLC*, independent of FRI[1]. This is achieved primarily through an RNA-mediated chromatin silencing mechanism involving alternative processing of a collection of non-coding *FLC* antisense transcripts, collectively named *COOLAIR*[22]. Use of a proximal polyadenylation site leads to recruitment of a histone demethylase (FLD) to the locus, which in turn leads to a reduction in H3K4me2 levels at *FLC*, resulting in suppression of expression[22]. Feedback from the chromatin state then reinforces choice of the *COOLAIR* proximal splice and polyA site, possibly through a kinetic coupling mechanism[23,24].

The result is a complex network of antagonistic interactions, centred on the *FLC* locus and involving a wide range of factors that act to establish expression prior to cold exposure, and through this, the need for vernalising cold in order to flower. This has important consequences for the life history of the plant as the initial levels of *FLC* determine the length of cold required in order to fully accelerate flowering, and thus has important implications with regards to aligning flowering with environmentally optimal conditions.

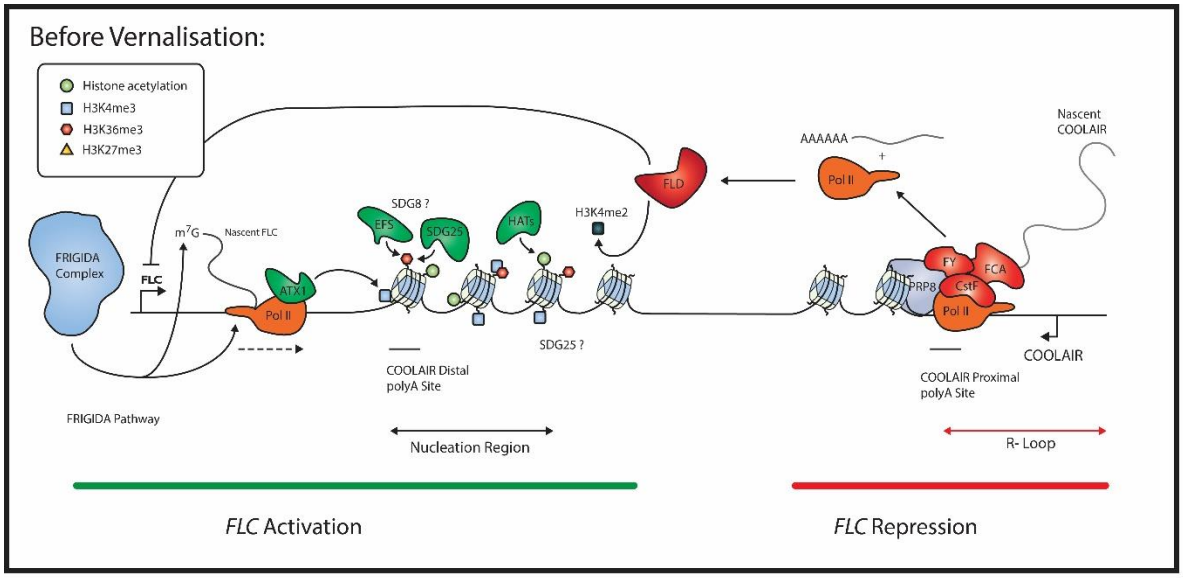

Figure 4: Illustrating the antagonistic inputs of the autonomous and FRIGIDA pathways in determining initial levels of *FLC* expression prior to cold exposure. The FRIGIDA pathway, responsible for activating *FLC* expression, acts to inrease the proportion of *FLC* mRNA that possess a 5' cap, as well as facilitating the recruitment of a number of chromatin modifying factors that increase the levels of histone modifications associated with high levels of transcription. By contrast, the autonomous pathway suppresses *FLC* expression: 3' processing of COOLAIR in an FCA dependent manner results in utilisation of COOLAIR's proximal polyA site, which in turn results in FLD-dependent reductions in H3K4me2 levels at the locus, resulting in suppression of expression.

1.4 Initial Shutdown and the Complexities of Sense-Antisense Transcriptional Circuitries.

During vernalisation, exposure to cold causes transcriptional downregulation of *FLC* expression within the first two weeks. This initial shutdown of *FLC* is mediated by the combinatorial action of two distinct pathways (Figure 5). Firstly, cold exposure results in recruitment of the protein VAL1 to B3 cis motifs found in a specific region of *FLC* intron 1 (termed the "nucleation region", see below). In turn, VAL1 recruits the histone deacetylase HDA19, leading to reduced histone acetylation and, consequently, transcription[25]. In tandem, expression of *COOLAIR* is also thought to contribute towards transcriptional shutdown- expression is highly induced by the cold, reaching maximum levels around 3 weeks, before decreasing upon return to warmth[26]. Together, these represent the earliest known events of the vernalisation process, and are thought to be essential for the subsequent establishment of epigenetically stable silencing of *FLC.*

Whilst VAL1's role has been well-resolved, the mode of action of *COOLAIR* by contrast remains more elusive, although mounting evidence supports a functional role. For example, addition of the *COOLAIR* promoter sequence to a reporter gene confers silencing of that reporter upon 2 weeks of cold exposure[26], and in the non-vernalised state, both 3' processing[22] and splicing[27] of these antisense transcripts have been shown to have functional consequences on *FLC* expression. With regards to the process of vernalisation, recent research has shown sense and antisense transcription

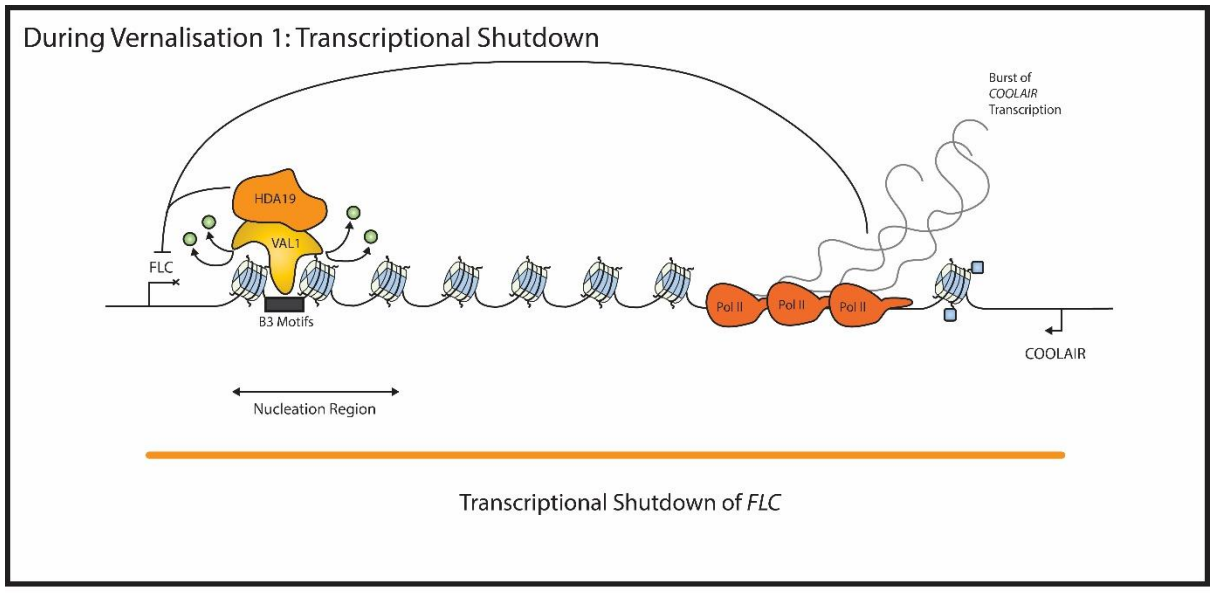

Figure 5: Illustration of the components mediating the initial transcriptional shutdown of *FLC* upon cold exposure. During the first two weeks of vernalisation, VAL1 is recruited to B3 cis-motifs contained within the nucleation region. This facilitates the recruitment of a variety of factors to the locus including the histone deacetylase HDA19, leading to decreased histone acetylation levels, a mark typically associated with active transcription. At the same time, expression of *COOLAIR* is induced by the cold, reaching its maximum levels around 3 weeks after cold exposure. Whilst its role remains thus far elusive, it is thought to assist in the transriptional shutdown of *FLC*, and the establishment of a transcriptional state permissive to the accumulation of repressive epigenetic marks.

of the *FLC* gene body to be anti-correlated, and apparently, mutually exclusive, providing a mechanism by which *COOLAIR* up-regulation might transcriptionally shut-down *FLC* (Rosa et al, in press), though how cold biases transcription in the anti-sense direction remains unclear. Though *COOLAIR* transcripts do not appear to be absolutely required for vernalisation[28], analysis of transgenic lines abrogated in *COOLAIR* production showed decreased rates of *FLC* down-regulation in the cold[29]. Thus, the initial transcriptional shutdown of *FLC* is mediated through a combination of both *COOLAIR* upregulation, as well as reductions to histone acetylation levels mediated by VAL1.

1.5 Altering the Balance: Coordinated Shifts in Chromatin Modifications at *FLC* Underpin Epigenetic Silencing of the Locus.

This initial transcriptional shutdown is followed by a cold-mediated recruitment of specific factors to the locus. The result is a coordinated deposition of the repressive epigenetic mark, H3K27me3, and loss of the activating mark H3K36me3, at a local nucleation region within the *FLC* gene. This region extends from the +1 to +4 nucleosome, covering exon 1 and the beginning of intron 1 (Figure 6). The dynamic reprogramming of this region, and replacement of H3K36me3 by H3K27me3 is sufficient to silence *FLC* expression[30]. Indeed, the two marks show opposing profiles during the vernalisation process, suggesting antagonistic roles that are likely necessary for enabling the switching between, and inheritance of, mutually exclusive epigenetic states[14].

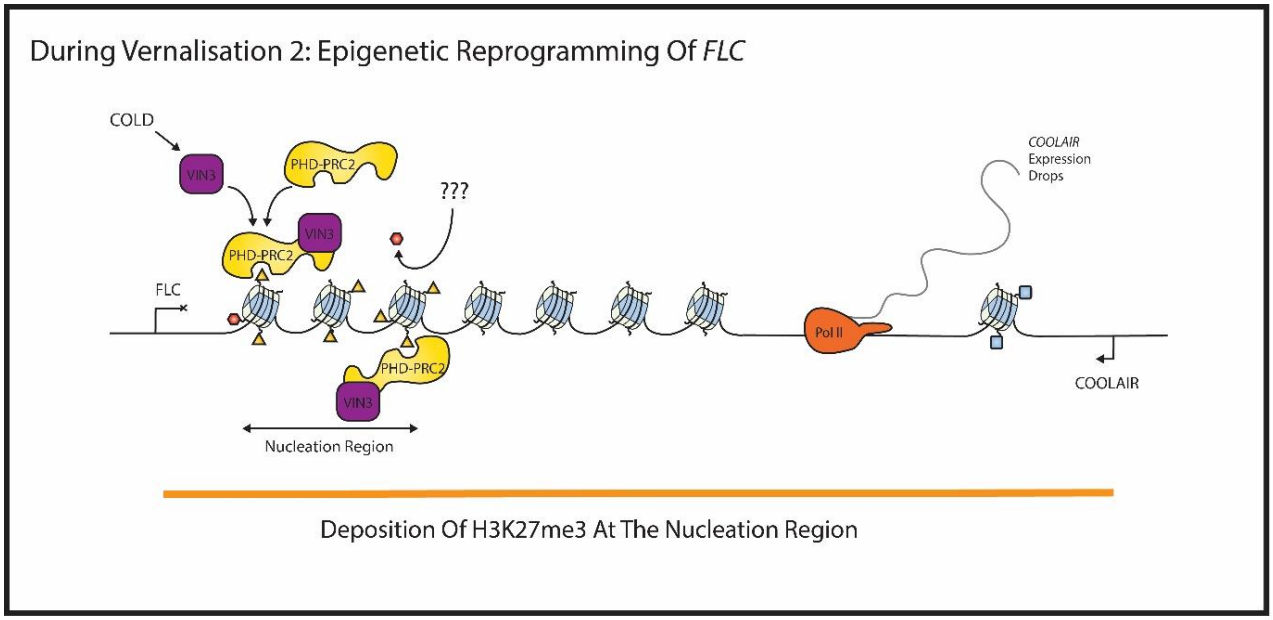

Figure 6: Schematic illustrating nucleation of the repressive epigenetic mark H3K27me3 at a specific region, the nucleation region within *FLC*. Following transcriptional shutdown of *FLC*, the cold controlled recruitment of various factors to the locus results in accumulation of H3K27me3 at the nucleation region: cold induces the expression of *VIN3* which is responsible for the recruitment of the complete PHD-PRC2 complex, which trimethylates H3K27. Concomitant with this, levels of the activating mark H3K36me3 decline, in a process mediated by a hitherto unknown factor. As these marks accrue, *COOLAIR* expression declines, reaching its minimum by about 4 weeks.

This localised deposition of H3K27me3 is mediated by a group of proteins that together form what is known as the PRC2 complex. Whilst the core PRC2 complex (composed of VRN2, SWINGER, FIE and MSI1) is constitutively associated with the *FLC* locus[30], cold exposure induces the expression of the PHD finger protein VIN3[31], which is thought to recruit its constitutively expressed homologue, VRN5, to the nucleation region, and whose association with the core PRC2 complex is thought to increase its histone methyltransferase activity[32]. Together, this complex mediates, and is necessary for, both the vernalisation response[33] and the accumulation of H3K27me3 at the nucleation region. Whilst the factors mediating H3K27me3 deposition are well resolved, the components underpinning H3K36me3 removal are so far unknown- *COOLAIR* appears to play a role however, with lines deficient in *COOLAIR* production showing reduced rates of H3K36me3 loss[34], with a concomitant reduction in the rate of *FLC* silencing. Thus the antisense appears to, in addition to or as part of its role in early transcriptional shutdown of *FLC*, help facilitate the coordinated switching of chromatin states at *FLC* during vernalisation.

1.6 Memory of the Cold and Stable Maintenance of Epigenetic Silencing.

Upon return to the warmth, the PRC2-PHD complex spreads across the entirety of the *FLC* locus, with a concomitant increase in H3K27me3 levels across the entirety of the gene body[30] (Figure 7). This spreading of H3K27me3 is required to maintain the epigenetic silencing of *FLC* during the multiple cellular divisions that underlie subsequent development upon return to the warmth[35]. This maintenance in turn is dependent on a number of factors, including VIN3, VRN2 and VRN5, and importantly, LHP1[36]. The latter is able to bind H3K27me3[37] and physically associates with the *FLC* locus

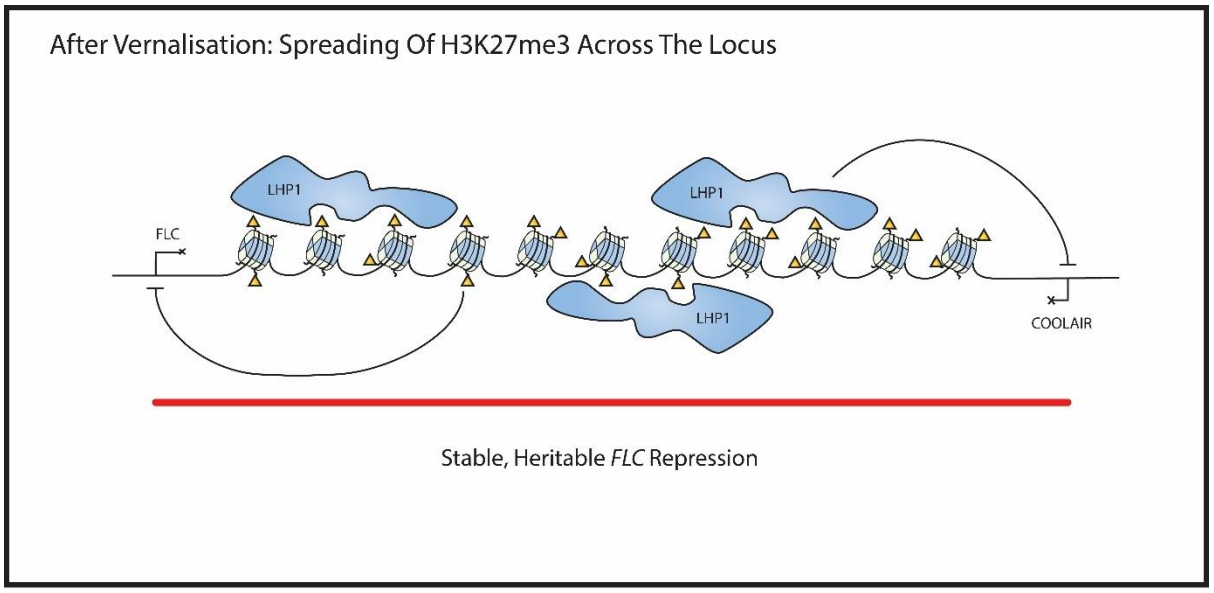

Figure 7: Schematic illustrating the spreading of H3K27me3 across the *FLC* locus. Upon return to the warmth, H3K27me3 spreads out from the nucleation region, across the entirety of the *FLC* locus. This is then bound by LHP1, a protein factor essential for maintenance of the repressive epigenetic mark at the locus. The result is stable, mitotically heritable epigenetic silencing of *FLC* and the establishment of competency to flower.

upon return to the warmth following cold exposure[38]. Together with EMF1 and a histone H3K4 demethylase, it forms a complex that plays a role in maintenance of the epigenetically silenced state through subsequent cell divisions. A similar role for LHP1 has been shown for *FT*, where it is also required to maintain repression[39].

1.7 Conserved Hub, Diverse Responses: *FLC* Centrality Underlies Significant Variation In the Vernalisation Response Across Different Accessions.

Despite significant conservation of both *FLC* and the associated trans-factors involved in the vernalisation response, significant variation exists both between and within different plant lineages in the way they respond to cold exposure. This is most notably seen for the period of cold required to establish full repression of *FLC* expression i.e. the vernalisation requirement. Within *Arabidopsis thaliana* accessions alone, for example, there is substantial diversity in the extent of cold exposure necessary to establish competency to flower[40]- whereas some accessions require as much as 12 weeks of cold exposure in order to fully accelerate flowering, others have dispensed with the requirement for vernalisation entirely and flower in the absence of cold.

This variation likely has adaptive consequences: *Arabidopsis thaliana* exhibits significant latitudinal variation in its distribution worldwide[41] and, in turn, such geographical diversity will expose different populations to very different climactic conditions. Specifically, it will mean that different populations are subjected to differing winter regimes. Given these environmental differences, what exactly constitutes an environmentally aligned flowering time will also vary and by extension so will the vernalisation requirements necessary to optimally time flowering. Thus, the diversity in vernalisation responses and requirements seen across *Arabidopsis* accessions globally makes sense given their wide and variable geographical distributions.

QTL mapping of the variation in vernalisation responses across *Arabidopsis* accessions first highlighted the importance of only a small number of loci, including *FLC,* as responsible for the diversity in vernalisation requirements seen[42,43]. Thus, despite its centrality in integrating temperature cues, variation at the *FLC* locus is sufficient to modulate and affect responses to temperature, with subsequent ramifications for flowering and flowering time.

Subsequent exploration of this functional diversity at *FLC* and analysis of the variation present at the locus across 1307 *Arabidopsis thaliana* accessions revealed the existence of multiple *FLC* haplotypes, each defined by distinct non-coding variation at the locus (despite almost complete coding sequence conservation), as well as differing vernalisation requirements[44]. Through comparisons of the sequence encoding *FLC* as well as 50kb either side of the *FLC* loci in each accession, a total of 20 haplotypes were

identified (Figure 8), 5 of which predominate in the worldwide population. Of these 5, 3 haplotypes are characterised by the requirement for long periods of cold to vernalise fully (slow vernalisers), and 2 are distinguished by their requirement for only short periods of cold to fully accelerate flowering (rapid vernalisers).

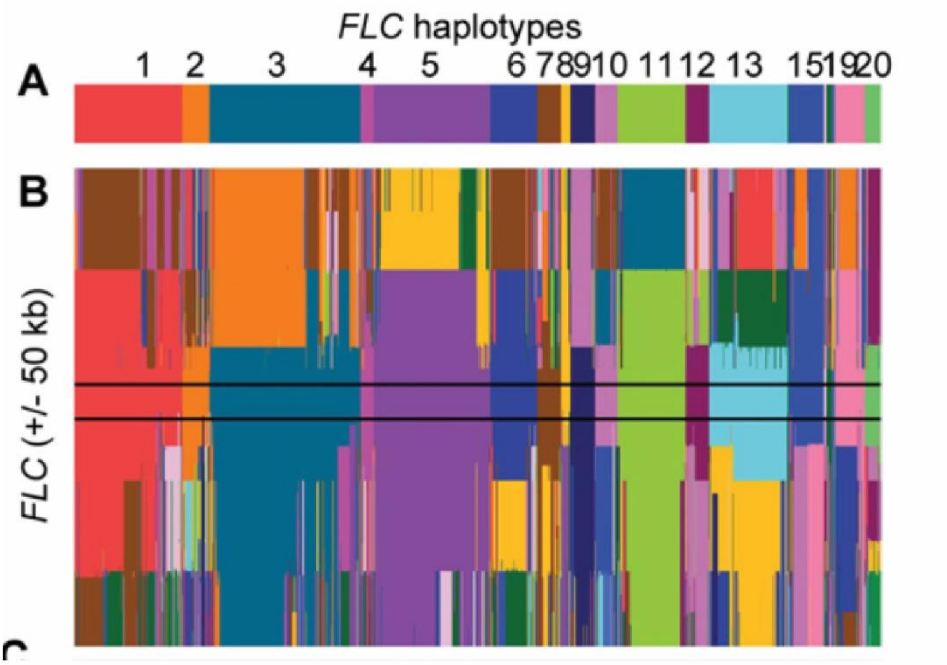

Figure 8: Figure from Li et al, 2014, illustrating the diversity present in the sequence of *FLC* and the surrounding region of 1307 worldwide *Arabidopsis thaliana* accessions. (A) Illustrates different *FLC* haplotypes, 20 in total, 5 of which predominate (1, 3, 5, 11 and 13). (B) Shows the haplotype structure of the *FLC* region as well as the surrounding 50kb in each of the 1307 accessions. At each SNP position, colours indicate the most likely haplotype membershio for each accession, as determined in the paper by fastPHASE analysis. Accessions are ordered according to the haplotype present at the *FLC* locus itself.

Further analysis of a number of accessions belonging to the 5 most prevalent haplotypes revealed that the distinctive vernalisation phenotypes identified were a product of altered *FLC* silencing dynamics, with the rate of accumulation of the repressive chromatin mark H3K27me3 appearing to differ between the accessions[45]. Thus, it appears that variation in the epigenetic silencing of *FLC* contributes to the natural variation in vernalisation responses observed across *Arabidopsis thaliana* accessions.

This was first analysed in detail for the slowly vernalising accession Lovvik-1 (Lov-1). An *Arabidopsis thaliana* accession from Northern Sweden, from the High Coast region at the Northerly limit of the *Arabidopsis* range, it was selected for study due to the extreme nature of its vernalisation requirement- it requires nearly 3 months at 5^{o}c to fully accelerate flowering, one of the longest vernalisation requirements observed across all accessions studied.

Analysis of the genetic basis of the slowly vernalising phenotype in this accession by Coustham et al[46] identified a region near the 5' end of *FLC* containing 4 non-coding single nucleotide polymorphisms (SNPs) sufficient for the distinct vernalisation requirement observed in Lov-1. This genetic variation was shown to quantitatively modulate PRC2 mediated epigenetic silencing, and influence the accumulation of the repressive H3K27me3 modification at *FLC* in response to the cold: specifically, it took 9 weeks to reach maximal levels of H3K27me3 compared to 4 weeks in the rapidly vernalising accession ColFRI.

Differences in the dynamic epigenetic reprogramming of *FLC* in response to cold across *Arabidopsis thaliana* accessions therefore appear important as a source of variation affecting vernalisation responses. However, this does not necessarily represent the sole way in which genetic diversity shapes and impacts vernalisation requirements. Recent work by Li et al[47], in which the slowly vernalising accession Var2-6 (also found in Northern Sweden, but belonging to a different haplotype group to Lov-1) was studied has implicated the role of altered antisense splicing in this accession's distinct vernalisation requirement. Thus, it appears that different aspects of the vernalisation mechanism might have the capacity to be modulated in order to achieve functionally similar adaptive alterations to the vernalisation requirement.

1.8 A Number of Questions Surrounding Natural Variation Remain Outstanding.

Although numerous advances have been made in the understanding of natural variation in vernalisation requirements, a number of questions remain outstanding, particularly with regard to which aspects of the molecular mechanism varies. In terms of Lov-1, which of the 4 polymorphisms contribute to the slowly vernalising phenotype remains unknown, as well as the nature of their influence on H3K27me3 dynamics. More broadly, whether different accessions display distinct silencing dynamics and kinetics has been comparatively under-explored.

The aim of this thesis is therefore to investigate the natural diversity in vernalisation that exists across *Arabidopsis thaliana* accessions. It will seek to better characterise the distinct vernalisation response of Lov-1, as well as analyse the vernalisation dynamics displayed by different accessions upon cold exposure. This will provide mechanistic insight into evolutionary processes underpinning adaptation.

1.9 Project Aims:

1. **Explore histone dynamics in Lov-1 and the rapidly vernalising Edi-0:** Whilst histone dynamics in Lov-1 have begun to be explored, previous efforts have only been at a low

spatial resolution, and only in comparison to a single rapidly vernalising accession, ColFRI. My work will extend this analysis to a second rapid vernalising accession, Edi-0, and to provide a better characterisation of *FLC* chromatin changes observed in Lov-1 (Chapter 3).

2. **Assess the importance of the +598 SNP and its contribution to the Lov-1 slow vernalising phenotype:** Previous work has narrowed down the causative variation of the Lov-1 phenotype to 4 SNPs. My work will assess the importance of a particular polymorphism, the so-called +598 SNP (relative to the transcriptional start site) in the distinct vernalisation requirement of Lov-1 (Chapter 4).

3. **Investigate *FLC* silencing dynamics in ColFRI, Lov-1 and Var2-6:** Characterizing expression dynamics of *FLC* before and during cold exposure has not been undertaken at high temporal resolution for many accessions. Such an analysis is necessary if our knowledge about the variation in the vernalisation mechanism across the accessions is to be effectively reconciled with the distinct vernalisation requirement/responses observed. My work will see such an experiment carried out, to demonstrate the impact of diversity in the vernalisation process on *FLC* silencing (Chapter 5).

Chapter 2- Materials and Methods.

2.1 Plant Materials and Growth Conditions.

Plants were grown as described previously in Coustham et al[46]. In all cases, seeds were surface sterilised, sown on MS media plates (no glucose) and stratified for 2 days at 5^{o}c. They were then grown for 10 days in long-day conditions (16 hours light at 20^{o}c, 8 hours darkness at 16^{o}c).

For non-vernalised plants, the plates were left in pre-growth for another 5 days and then material harvested directly. For vernalised plants, following the pre-growth, the plates were transferred to 5^{o}c under short-day conditions (8 hours light, 16 hours darkness). Vernalisation at higher temperatures (8^{o}c and 12^{o}c) was carried out under the same short-day conditions. T0 seedlings were harvested immediately off the plates after vernalisation. T10 seedlings were grown for a further 10 days in the warmth after transfer from the cold. T30 seedlings were transferred from plates to soil at the time of the T10 harvest, and were grown for another 20 days in the warm.

2.2 Flowering Time Analysis.

Flowering time was assayed by counting the number of days from the end of the vernalisation period to the first sign of a bud having appeared in the middle of the rosette of an individual plant.

2.3 qPCR Expression Analysis.

Expression analysis was performed as described in Coustham et al, 2012[46]: cDNA was synthesised from extracted RNA using SuperScriptIII (Invitrogen) using gene specific primers previously used by the lab[47] and analysed by qPCR on a LightCycler 480 II instrument (Roche) using LightCycler 480 SYBR Green mix (Roche). Results were normalised against the *Arabidopsis UBC* gene (*At*5g257560) whose expression is known not to change with cold exposure.

2.4 ChIP Assay.

The assay used to assess histone modification levels were performed as previously described in Yang et al, 2014[14]: briefly, nuclei were extracted using Honda buffer, and subsequently sonicated for 15 minutes (3x 5 minutes, 30 seconds on, 30 seconds off). This was followed by DNA recovery and purification and then immunoprecipitation (IP). For the IP, the following antibodies were used: anti-Histone H3 (trimethyl K27) antibody (Millipore, 07-449), anti-Histone H3 antibody (Abcam, ab1791), and anti-Histone H3 (trimethyl K36) antibody (Abcam, ab9050) and expression analysis carried out as for the qPCR expression analysis, using standard primers used by the lab and which have been

previously described[47]. Raw results for H3K27me3 and H3K36me3 levels were first normalised to H3 levels, then to input controls, and then finally to control genes whose levels of H3K27me3/H3K36me3 do not change with cold. For H3K36me3, this is *Actin*. For H3K27me3, this is *STM*.

Chapter 3: Role of non-coding variation in specifying *FLC* haplotype behaviour.

3.1 Background.

Recent analyses investigating the role of non-coding diversity at the *FLC* locus in different accessions identified variation in the nature of chromatin dynamics during vernalisation as the driver of distinct vernalisation phenotypes in two different accessions. Comparisons of H3K27me3 accumulation in the slowly vernalising Lov-1 and the rapidly vernalising ColFRI suggested quantitative variation in the deposition of the mark as the driver of their distinctive vernalisation phenotypes- specifically, accumulation of the modification appeared to be slower in Lov-1.

A small region surrounding the transcriptional start site of *FLC* containing 4 SNPs was identified as causative of this phenotype, with the SNPs suggested to be quantitatively modulating the rate of accumulation of H3K27me3 in this region[46]. This work thus identified diversity in the epigenetic reprogramming of *FLC* as a means of generating diversity in vernalisation requirement.

However, a number of questions remained outstanding. Firstly, the initial analysis of the accumulation of H3K27me3 at *FLC* in both accessions was undertaken at a spatial resolution that provided only limited coverage of the *FLC* locus. Thus, the exact nature and way in which the dynamics differed between the accessions needed to be fully elucidated. Additionally, the rapidly vernalising line used for comparative purposes, ColFRI, is not a bona fide natural accession, instead representing a line of the accession Col-0 (which lacks a vernalisation requirement) in which *FRI* has been introgressed in to generate a vernalisation requirement. Therefore, a comparison to a genuine naturally occurring rapid vernaliser was required in order to establish whether these differences have relevance with regard to natural populations. To that end, a high spatial resolution chromatin immunoprecipitation (ChIP) experiment was undertaken in order to assess the H3K36m3 and H3K27me3 profiles in Lov-1 and the naturally occurring rapid vernaliser, Edi-0, during vernalisation.

Use of Edi-0 for this purpose holds interest beyond simply its utility for comparative purposes. In terms of the 4 SNPs identified in Coustham et al (SNPs -326, -56, +121 and +598 relative to the transcriptional start site) as holding potential functional relevance, Edi-0 is

more genetically similar to Lov-1 than ColFRI, differing at only 2 of the 4 positions (-56 and +598). Thus, comparative analysis of Lov-1 and Edi-0 will further inform efforts to establish which of these SNPs possess functionality.

3.2 Aim.

Investigate histone modification dynamics in the rapidly vernalising accession Edi-0 and the slowly vernalising Lov-1.

3.3 Results.

3.3.1 H3K27me3 profiles differed between Lov-1 and Edi-0 upon return to the warmth (Figure 1A and 1B): Edi-0 and Lov-1 displayed similar, low levels of H3K27me3 prior to cold exposure (Figure 1C, paired t-test, $p > 0.5$). This continued into the cold, with both accessions demonstrating similar patterns of accumulation of this repressive chromatin mark during exposure and each showing a clear peak of H3K27me3 just downstream of the transcriptional start site (TSS), in the nucleation region (Figure 1D). This feature is well established in the literature, and is in-keeping with previous analyses of the epigenetic dynamics at *FLC* during cold exposure[14]. The rate of accumulation of H3K27me3 did not differ between these accessions during this period ($p > 0.5$). Upon return to the warmth, both accessions showed signs of limited spreading of H3K27me3 beyond the nucleation region, as evidenced by a rise in levels across the gene body 10 days after return to the warmth (Figure 1E), with the repressive mark no longer delimited to the region immediately following the TSS. The difference in this case was also non-significant ($p > 0.5$). However, profiles differed by 30 days after return to the warmth (Figure 1F, $p < 0.001$): Edi-0 showed increased spreading of the modification across the gene body, with a rise in levels compared to previous timepoints. Lov-1 by contrast showed a marked reduction in H3K27me3. Thus, differences in stability of the H3K27me3 modification upon return to the warmth appear to drive the differential epigenetic profiles observed between the two accessions, as opposed to differing rates of accumulation during the cold.

3.3.2 H3K36me3 dynamics also differed between the two accessions upon return to the warmth (Figure 2A and 2B): Given these observations, it was asked whether such a difference would also be seen in the antagonistic chromatin mark, H3K36me3. Levels in

non-vernalised plants were high in both accessions (Figure 2C) and did not differ from one another ($p > 0.5$), reflecting the association of the mark with active transcription and in-keeping with previously published data[14]. Levels decreased upon cold exposure (Figure 2D) although to differing extents: whilst Edi-0 demonstrated a clear drop in levels in the nucleation region, the extent of the decrease was far smaller in Lov-1 and appeared to be localised to the gene body, although this difference between the two accessions was not significant ($p = 0.25$). Levels remained similar between the accessions 10 days after return to the warmth ($p = 0.07$, Figure 2E). By 30 days after return to the warmth however, the profiles differed (Figure 2F, $p < 0.01$). In Edi-0, H3K36me3 levels continued to drop whereas, in Lov-1, no such drop was observed, with H3K36me3 levels similar to that observed at 10 days in the warm. One might postulate that if the analysis duration were extended, H3K36me3 levels in Lov-1 would begin to rise, concomitant with the aforementioned loss of H3K27me3 seen by day 30 in this accession.

Interestingly, the decline in H3K36me3 levels appeared to lag behind the gain of H3K27me3. Whereas a sharp nucleation peak of H3K27me3 was observed directly after cold exposure, H3K36me3 levels did not show a reduction until later, at 10 (Lov-1 and Edi-0) and 30 (in Edi-0) days after return to the warmth. Additionally, the data suggests different dynamics between the two accessions, with Lov-1 H3K36me3 levels not dropping significantly during cold exposure itself, only upon the return to warmth, and even then, only transiently. By contrast, they show a consistent and concerted drop in Edi-0 upon cold exposure. Thus, comparisons between the H3K36me3 profiles of the two accessions reveal that, as for H3K27me3, the most striking differences occur 30 days after the return to warmth following vernalisation, where the profiles diverge significantly, correlating with the differential *FLC* expression profiles observed (see below).

3.3.3 Differential chromatin profiles between the accessions correlated with differences in unspliced and spliced *FLC* levels: Given the differences observed in the chromatin profiles at *FLC* in the two accessions, it was next asked whether these differences held consequences for expression of *FLC*, both during and after the cold. Expression of unspliced *FLC* dropped for both Lov-1 and Edi-0 upon cold exposure, with no difference either before vernalisation or immediately after (Figure 3A, t-test, $p = 0.228$ and $p = 0.296$ respectively). However, contrasting profiles between the two accessions were observed for 10 and 30 days after

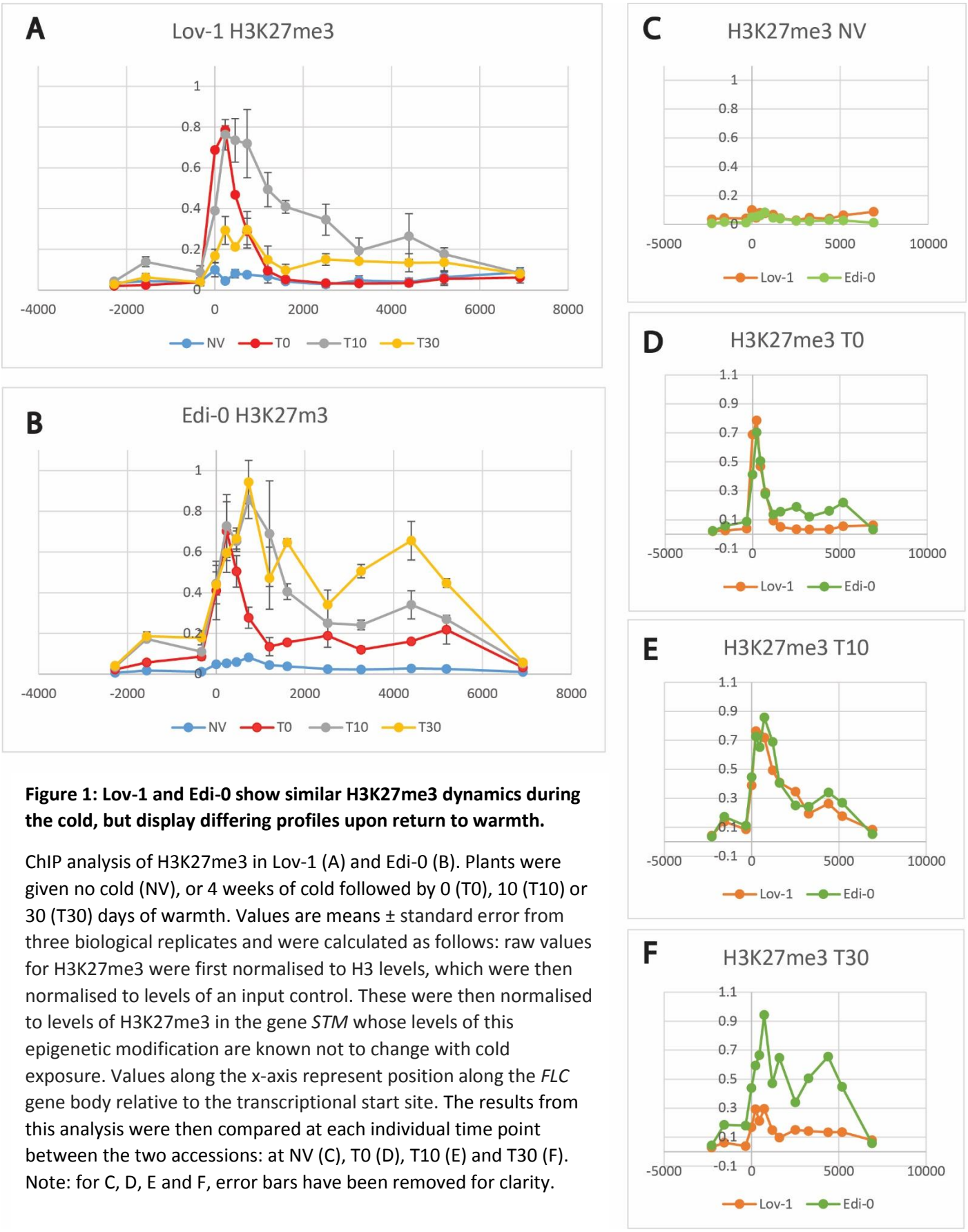

Figure 1: Lov-1 and Edi-0 show similar H3K27me3 dynamics during the cold, but display differing profiles upon return to warmth.

ChIP analysis of H3K27me3 in Lov-1 (A) and Edi-0 (B). Plants were given no cold (NV), or 4 weeks of cold followed by 0 (T0), 10 (T10) or 30 (T30) days of warmth. Values are means ± standard error from three biological replicates and were calculated as follows: raw values for H3K27me3 were first normalised to H3 levels, which were then normalised to levels of an input control. These were then normalised to levels of H3K27me3 in the gene *STM* whose levels of this epigenetic modification are known not to change with cold exposure. Values along the x-axis represent position along the *FLC* gene body relative to the transcriptional start site. The results from this analysis were then compared at each individual time point between the two accessions: at NV (C), T0 (D), T10 (E) and T30 (F). Note: for C, D, E and F, error bars have been removed for clarity.

return to the warmth, with unspliced *FLC* levels in Lov-1 rising by 10 days, and continuing to rise until 30 days after removal from cold, reaching approximately 3x the levels observed immediately following cold exposure (Figure 3B). By contrast however, expression of unspliced *FLC* in Edi-0 continued to drop over the same period, evidenced by a T30/T0 ratio of <1. For both time points, the difference between unspliced *FLC* levels for the accessions was significant ($p = 0.03$ for T10 and $p < 0.01$ for T30). This differential reactivation and repression of expression seen in Lov-1 and Edi-0 respectively correlates with and supports a functional role for the differential H3K27me3 dynamics observed upon return to the warmth, and suggests that differences in epigenetic stability of the H3K27me3 modification in the warmth between the two accessions could account for differences in the vernalisation phenotypes observed.

As with unspliced *FLC*, levels of spliced *FLC* transcripts in non-vernalised plants, as well as those vernalised for 4 weeks did not differ significantly from each other (Figure 3C, $p = 0.48$ and $p = 0.2$ respectively). The profiles diverged 10 days after return to the warmth following the cold however, whereupon significantly differential dynamics are observed ($p = 0.03$). In the same manner as unspliced *FLC*, Lov-1 spliced *FLC* levels began to increase over this period, whilst in Edi-0 they continued to drop. Interestingly, this significance was lost by day 30 ($p = 0.1$), with reactivation of expression being observed in both Lov-1 and Edi-0, although to greatly differing extents. Whereas spliced *FLC* levels in Lov-1 at this timepoint reached 10x the amount observed immediately after vernalisation, levels in Edi-0 only reached approximately 1.5x (Figure 3D). This significant and stable decrease in *FLC* expression in Edi-0 correlated with the induction of *FT* expression, with levels significantly higher than Lov-1 30 days following return to the warmth (Figures 3E and 3F, $p = 0.04$).

3.3.4 Spliced and unspliced *FLC* levels showed distinct expression patterns: Intriguingly, the profiles of spliced and unspliced *FLC* are not entirely coherent. Specifically, the magnitude of expression reactivation observed was far greater for spliced than unspliced *FLC*. This is notably seen in Lov-1, where, comparisons of T30/T0 ratios highlight a reactivation almost threefold higher in the spliced than the unspliced. Perhaps even more markedly, in Edi-0, whereas unspliced levels continue to drop after cold exposure, levels of the spliced transcript appear to reactivate somewhat (although only slightly). This points to additional, non-transcriptional modes of control of spliced *FLC* accumulation, modes of control that

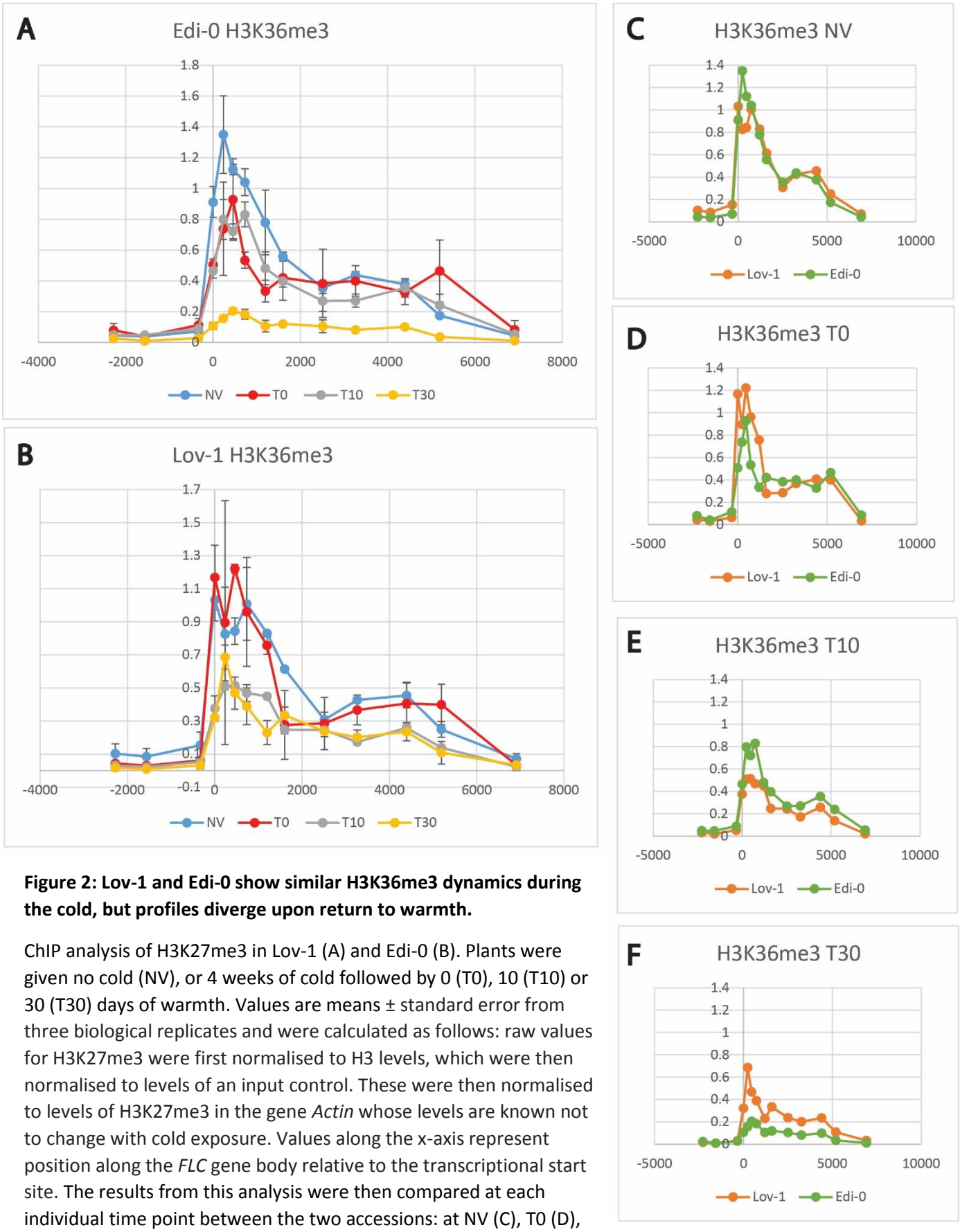

Figure 2: Lov-1 and Edi-0 show similar H3K36me3 dynamics during the cold, but profiles diverge upon return to warmth.

ChIP analysis of H3K27me3 in Lov-1 (A) and Edi-0 (B). Plants were given no cold (NV), or 4 weeks of cold followed by 0 (T0), 10 (T10) or 30 (T30) days of warmth. Values are means ± standard error from three biological replicates and were calculated as follows: raw values for H3K27me3 were first normalised to H3 levels, which were then normalised to levels of an input control. These were then normalised to levels of H3K27me3 in the gene *Actin* whose levels are known not to change with cold exposure. Values along the x-axis represent position along the *FLC* gene body relative to the transcriptional start site. The results from this analysis were then compared at each individual time point between the two accessions: at NV (C), T0 (D), T10 (E) and T30 (F). Note: for C, D, E and F, error bars have been removed for clarity.

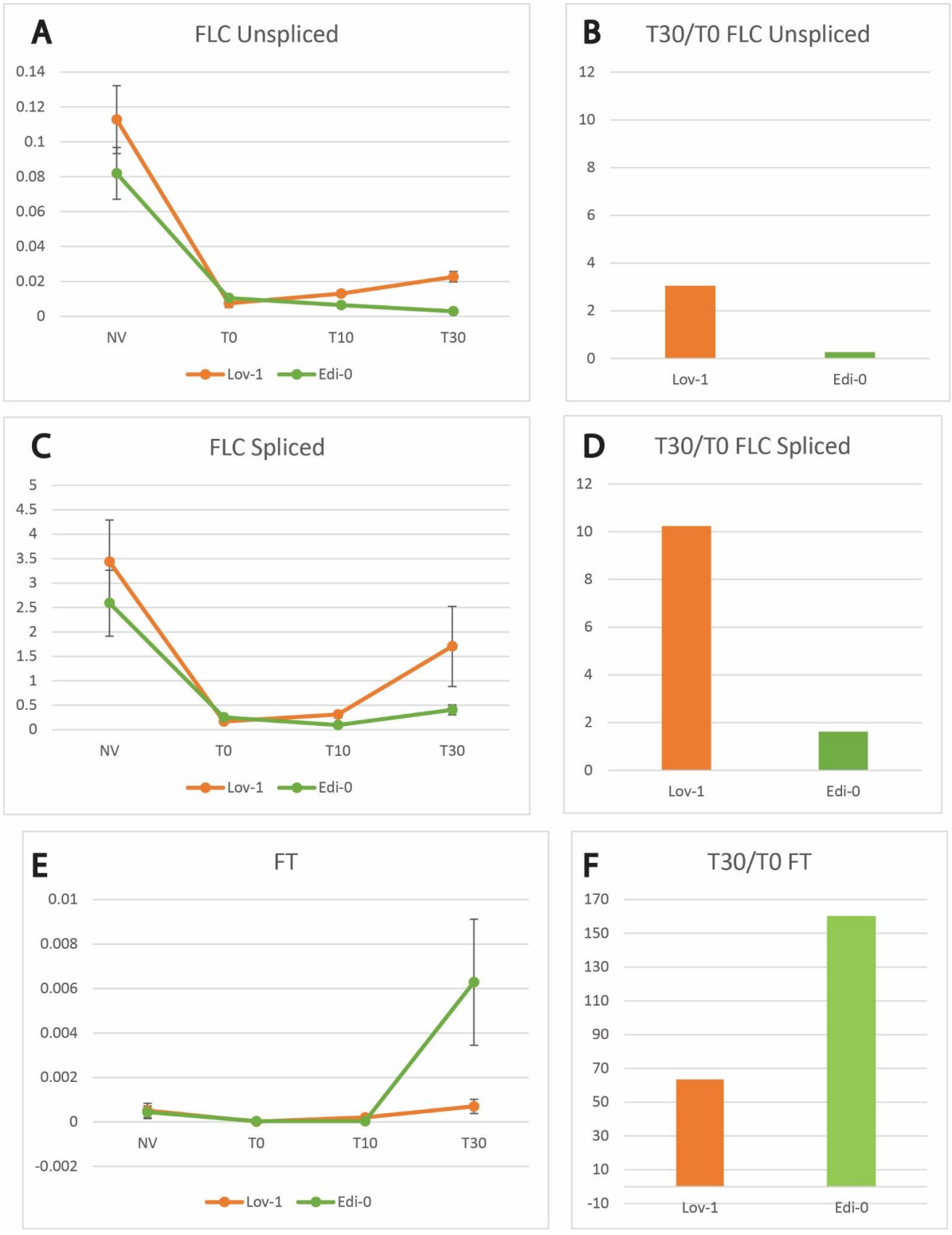

Figure 3: Lov-1 and Edi-0 show distinct expression patterns of the floral genes, *FLC* and *FT* after 4 weeks vernalisation. A) Spliced *FLC* expression of non-transgenic Lov-1 and Edi-0 accessions. Plants were given either no cold (NV), 4 weeks of cold (T0) or 4 weeks of cold followed by 10 days of warmth (T10) or 30 days of warmth (T30). Values are mean ± standard error from three biological replicates, and are all normalised to UBC expression. B) The ratio of *FLC* spliced expression at T30 compared to T0. C) As for A, except that it is for unspliced *FLC*. D) As for B, except for unspliced *FLC*. E) As for A, except for *FT*. F) As for B, except for *FT*.

appear to be differentially regulated by temperature, due to their apparent absence in the cold and presence upon return to the warmth.

3.4 Discussion.

The main point to arise from this analysis is that H3K27me3 profiles appear broadly similar across the two accessions before and during the cold. Both accessions have similar basal levels of H3K27me3 before cold exposure, and appear to accumulate H3K27me3 in the nucleation region at a similar rate during the cold. This similarity is maintained initially upon return to the warmth, with both showing evidence of spreading of the epigenetic mark across the locus, in keeping with the dynamics observed in the rapidly vernalising, and well explored, ColFRI[14,30]. However, by 30 days after return to the warmth, the dynamics diverge, with Lov-1 demonstrating marked loss of H3K27me3, concomitant with the reactivation of *FLC* expression. Edi-0, by contrast, shows increased spreading, and establishment of stable epigenetic silencing, reflected in the lack of significant reactivation of *FLC* expression. Thus, modulations to the stability of this epigenetic modification upon return to the warmth, rather than quantitative variation in the accumulation of H3K27me3 during the cold, appears to drive the differential vernalisation phenotypes observed between the accessions, something that was revealed due to the higher spatial resolution achieved in this set of ChIP experiments. Additionally, the fact that these differential dynamics are observed, along with the lack of *FLC* reactivation, in Edi-0, provides supporting evidence specifically to the role of either the -121, the +598, or both SNPs in underpinning the distinct vernalisation characteristics possessed by the two accessions.

Another point of note to arise from this analysis is that, despite the fact that both are rapidly vernalising, ColFRI and Edi-0 possess quite distinct H3K27me3 profiles. Previous measurements of H3K27me3 at the nucleation region in ColFRI have identified far higher basal non-vernalised levels than that observed here for Edi-0[14]. Therefore the short vernalisation requirement seen in ColFRI appears to be a product of high starting H3K27me3 levels, differing dramatically from Edi-0, which appears to achieve this through increased stability of the mark following its deposition (compared to Lov-1). Thus, this analysis tentatively points to convergent evolution of rapid-vernalising life-histories, characterised by different patterns of non-coding variation, which in turn affect the vernalisation process

in different ways to achieve this shared life-strategy. However, it is important to remember that ColFRI does not represent a bona fide rapidly vernalising accession, and thus analyses of other, naturally occurring, rapid vernalisers are required in order to confirm this.

Finally, expression analyses highlighted differential dynamics observed between the spliced and unspliced profiles in both accessions. In Lov-1, the degree of reactivation seen in spliced *FLC* (a tenfold increase in expression from between the return to warmth and 30 days subsequent, representing a rise to almost 50% of starting-levels) was far greater than that observed in the levels of unspliced *FLC* (only a 3-fold rise over that same period, rising to only about 20% of initial levels). Similarly, the small degree of reactivation observed in spliced *FLC* expression in Edi-0 was not matched in the unspliced, where levels continued to drop during that same timeframe. An explanation for this might be an increase in splicing/co-transcriptional processing efficiency upon return to the warmth that is masking a reactivation of unspliced *FLC* transcription by increasing the rate of conversion of unspliced to spliced, and warrants further investigation.

3.5 Future Experiments.

3.5.1 ChIP Analysis Of Other Accessions: Further ChIP analysis to assess histone modification dynamics before, during and after cold exposure in a number of other accessions, namely, representative ecotypes from the other primary haplotypes previously identified[44] would be highly instructive. It would allow the elucidation of whether the impaired H3K27me3 stability seen in Lov-1 is unique to the accession or representative of a more broadly conserved strategy employed to yield a slow vernalisation phenotype. Additionally, exploration of epigenetic diversity at *FLC* across a wide variety of accessions would elucidate other aspects of the vernalisation mechanism subject to modulation and variation- whilst H3K27me3 dynamics appear to differ between Lov-1 and Edi-0, other accessions might have achieved the slowly vernalising phenotype in entirely different ways independent of this chromatin modification. Thus a wider exploration of the diversity between accessions will be greatly informative in highlighting which specific aspects of vernalisation are modulated and subject to variation.

3.5.2 Utilising CRISPR/Cas9 To Achieve A Clean *FLC* Deletion: Performing ChIP on wild-type accessions is relatively simple, due to the presence of only a single copy of *FLC*. However,

ChIP analyses of transgenic lines produced by the lab is significantly hampered by the fact that the *FLC* mutant used, *flc-2* (a fast-neutron allele mutant) still contains a sizeable portion of the locus, and has been subjected to a substantive genomic rearrangement. Thus, ChIP analysis of transgenic *FLC* copies artificially inserted into this background are complicated by the presence of this non-functional *FLC* copy.

To this end, and to circumvent this problem, I have produced, using the Golden Gate technique, 4 constructs, containing different pairs of sgRNAs targeting regions flanking the *FLC* locus, as well as Cas9 driven under the *UBI10* promoter. These have been transformed into *Arabidopsis thaliana*, the seed has been collected and sown, and we are currently awaiting the results of selection by basta spraying. If we manage to produce a clean, heritable deletion, it will make analysis of transgenic *FLC* lines, particularly for ChIP far easier. This will be particularly important in future work examining the comparative importance of cis and trans variation in producing the distinct vernalisation phenotypes seen across the accessions, as such an analysis that will make significant use of lines lacking endogenous copies of *FLC*.

Chapter 4: Analysis of Transgenic Chimeric Lines To Assess Function Of The +598 SNP In Lov-1.

4.1 Background.

Analysis of the chromatin profiles of *FLC* in Lov-1 and Edi-0 revealed, as in the original comparison between ColFRI and Lov-1, clear differences in the dynamics of the two principal histone modifications, H3K27me3 and H3K36me3. Unlike the previous analysis however, the results presented in Chapter 3 identified altered H3K27me3 stability in Lov-1, a feature which manifests as its loss from the locus upon return to the warmth, as the putative feature underpinning the accession's slowly vernalising phenotype. Indeed, loss of this mark from the locus coincides with reactivation of *FLC* expression, pointing to altered epigenetic stability, rather than differences in rate of accumulation of H3K27me3, as the driver of the distinct vernalisation requirements observed between the two accessions.

Whilst the mechanism underpinning the different vernalisation requirements between Lov-1 and Edi-0 was better resolved, the exact genetic variation underpinning this difference in phenotype still remained outstanding. Of the 4 SNPs first identified by Coustham et al, it was still unclear which, and in what combination, have functional effects that influence vernalisation requirements. Analyses of 192 *Arabidopsis thaliana* accessions revealed that the +598 SNP is found in only 2 accessions (Lov-1 and its closely related counterpart, Lov-5) and moreover, it was the sole polymorphism to significantly associate with flowering time in vernalising conditions. Additionally, the differential chromatin dynamics between Lov-1 and Edi-0 (which only differ in the -121 and +598 SNPs out of the 4) presented in Chapter 3 also supports the notion that the +598 SNP has a functional role- although is important to note that the effect of other polymorphisms unique to Edi-0 cannot be precluded and could easily confound the analysis. Irrespective, together, these separate lines of evidence supported the importance of the +598 SNP in production of the slowly vernalising Lov-1 phenotype.

In order to assess the importance of the +598 SNP chimeric FLC constructs were generated and *FLC* dynamics analysed in transgenic lines. Briefly, the *FLC* gene region was divided into three sections: the first spanning the promoter region up until approximately 400bp before the transcriptional start site (TSS), the second spanning the TSS as well as the entirety of the

first exon, and some of the first intron (and notably including all 4 SNPs identified by Coustham et al) and the third spanning the entirety of the rest of the gene. Whole *FLC* genes were constructed using different permutations of these sections, and then, in a subset of constructs, the SNP at position +598 was converted from that of one accession to another through site directed mutagenesis, in order to directly assess its effect.

In total 4 constructs had been made (by a previous post-doctoral researcher, Figure 1E)-

- LEL: possessing the first and third domains from Lov-1, but with the middle section (and hence the 4 SNPs) from Edi-0.
- ELE: the reciprocal of LEL, containing the 4 Lov-1 SNPs in the second domain.
- LEL +598: Identical to LEL save for the +598 SNP which was converted via site directed mutagenesis (SDM) to a "T" as in Lov-1.
- ELE +598: Identical to ELE, except for the +598 SNP which was converted to an "A" as in Edi-0 via SDM.

Comparisons of ELE with LEL +598 would therefore allow establishment of whether introduction of the +598 SNP alone would be sufficient to elicit *FLC* reactivation upon return to the warmth

4.2 Aim.

- To assess the functional importance of the +598 SNP in the Lov-1 slowly vernalising phenotype.

4.3 Results.

4.3.1 Possession of the Lov-1 variant of the +598 SNP is sufficient to generate *FLC* reactivation upon return to the warmth following vernalisation: Analysis of spliced *FLC* levels highlighted differences between the lines in *FLC* expression dynamics upon return to the warmth (Figure 1A). Whilst all showed significant drops in *FLC* expression during cold exposure, lines that possessed the Lov-1 +598 SNP, namely LEL +598 and ELE, had increased levels of *FLC* 30 days after return to the warmth compared to only 10 days after, indicating that some degree of reactivation of expression had occurred (Figure 1C). For LEL +598,

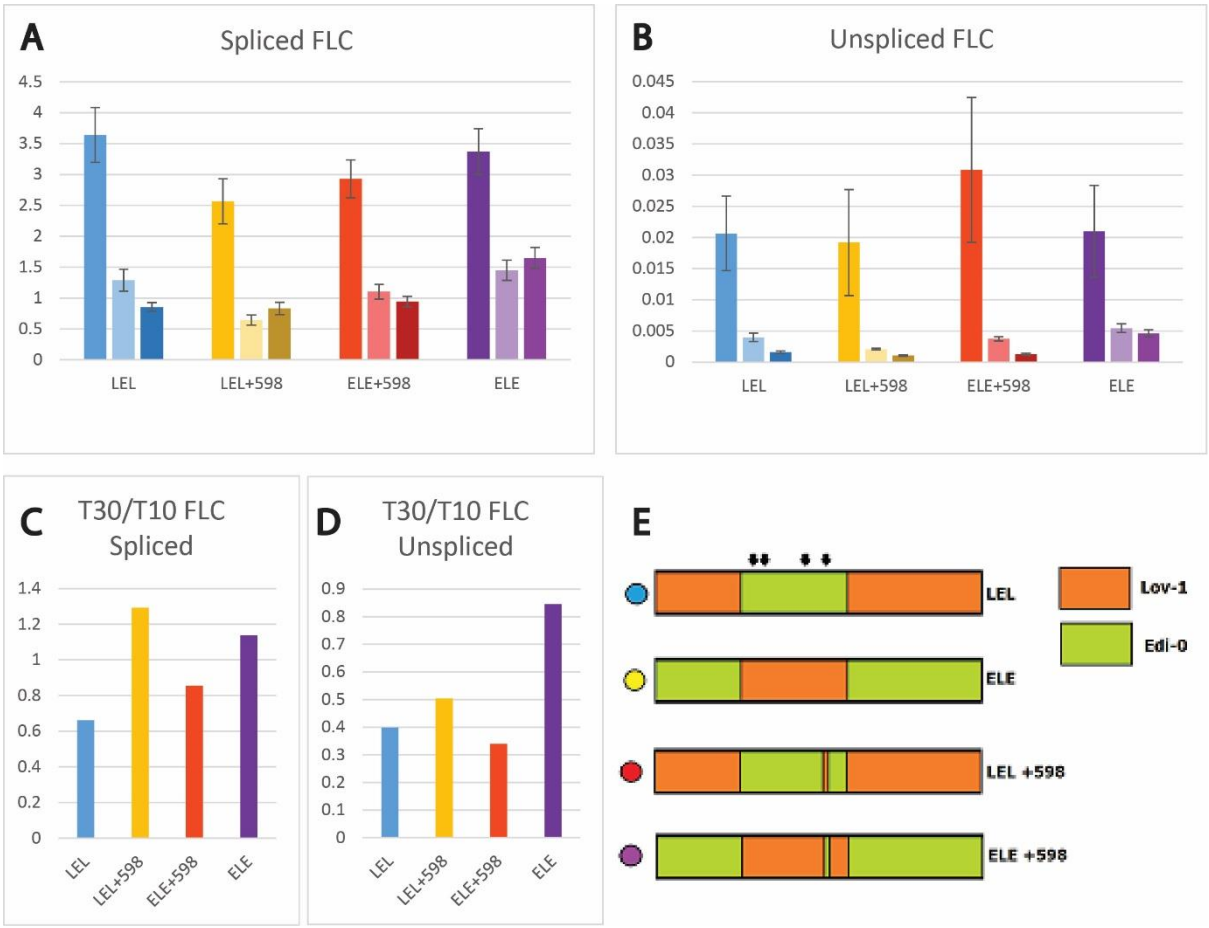

Figure 1: Transgenic analysis highlights the single Lov-1 +598 SNP alone as sufficient to produce *FLC* expression reactivation upon return to the warmth following cold exposure.

A) Spliced *FLC* expression analysis of pools of approximately 50 transgenic *FRI flc-2* plants for each line containing chimeric *FLC* alleles. Plants were given either no cold (NV, first bar per transgenic line), 4 weeks of cold followed by 10 days of warm (4WT10, second bar) or 30 days of warm (4WT30, third bar). Values are mean ± standard error from three biological replicates, and are all normalised to *UBC* expression. B) As above, except expression of the unspliced *FLC* transcript was measured. C) The ratio of spliced *FLC* expression at 4WT30 compared to 4WT10. D) The ratio of unspliced *FLC* expression at 4WT30 compared to 4WT10. E) Schematic illustration of the chimeric *FLC* constructs, composed of alternate combinations of parts of the *FLC* locus from Lov-1 (orange) and Edi-0 (green). Arrows represent the location of the 4 polymorphisms identified as putatively important in Coustham et al.

expression at 30 days following return to the warmth was 1.3x greater than after 10 days of warmth, and in ELE, this was 1.15x.

By contrast, the two lines with the Edi-0 +598 SNP showed no reactivation of *FLC* expression. Instead, expression dropped over the same time period- levels of spliced *FLC* in LEL at 30 days post-warmth were 65% of the expression at 10 days after return to the warmth, and in ELE +598, expression dropped 15% over the same period.

This indicates that, of the 4 SNPs initially highlighted by Coustham et al, only the +598 SNP is necessary in this context to produce reactivation of *FLC* expression following vernalisation. That reactivation of expression was not observed in ELE +598 but seen in ELE highlights the dispensability of the -56 SNP for *FLC* reactivation, and definitively establishes the +598 SNP as the causative non-coding cis variation at *FLC* responsible for the reactivation of expression seen in Lov-1.

4.3.2 The magnitude of reactivation of *FLC* expression is dependent on the genetic context: A difference in the magnitude of reactivation of *FLC* expression was observed between LEL +598 and ELE. This points to functionality of other polymorphisms not within the region initially identified by Coustham et al, and a contribution outside of the +598 SNP, in either the first or third domain. One possible explanation may be a number of polymorphisms present in the promoter region of Lov-1. Re-analysis of results in Coustham et al highlighted a possibly previously missed contribution of SNPs in this region to reactivation, and thus explorations of the genetic diversity in this region warrants further investigation.

4.3.3 Spliced and unspliced *FLC* levels showed distinct patterns of expression across all four lines analysed: Whilst reactivation of expression was seen in the spliced *FLC* profiles of lines carrying the Lov-1 +598 SNP, the same phenomenon was not observed during analyses of unspliced *FLC* levels (Figure 1B). Instead, all 4 of the lines examined showed declines in *FLC* expression between 10 and 30 days after return to the warmth, evidenced by T30/T10 ratios of <1 for all 4 lines (Figure 1D). However, this decrease in expression over that period was not equal across the lines. Expression of *FLC* in LEL dropped 60%, whilst expression in LEL +598 and ELE +598 dropped by 50% and 65% respectively in the same time period. Interestingly, expression of unspliced *FLC* in ELE only dropped by 17% in that time. Perhaps

most notably, a similar phenomenon was seen in Chapter 3, where a similar disparity in spliced and unspliced *FLC* profiles was also observed.

4.4 Discussion.

These results establish the involvement of the +598 SNP in affecting the propensity of spliced *FLC* expression to reactivate upon return to the warmth following vernalisation. Both of the transgenic lines possessing the Lov-1 +598 SNP, namely ELE and LEL +598, showed reactivation of spliced *FLC* expression, as evidenced by T30/T10 ratios greater than 1. By contrast, the opposite was seen in the two transgenic lines that possessed the Edi-0 +598 SNP (ELE +598 and LEL), where a decrease in spliced *FLC* expression between 10 and 30 days following cold exposure was instead observed (producing T30/T10 ratios less than 1). Although none of the differences observed were statistically significant, transgenic variability in terms of expression due to differing genomic contexts of the insertion has previously been identified as an issue when dealing with transgenic constructs containing *FLC*[48]. Thus the presence of the observed trends, trends that are in-keeping with previously published literature, despite the significant noise contained within each pooled transgenic population analysed, supports a functional role for the +598 SNP.

Additionally, comparisons of the two reactivating transgenics LEL +598 and ELE highlight a possible contribution of the promoter SNPs- indeed, re-analysis of the data collected and featured in Coustham et al supports such an association. The magnitude of reactivation of spliced *FLC* expression was greater in LEL +598 (which possesses the Lov-1 versions of these promoter-based polymorphisms) than in ELE (which possesses the Edi-0 equivalents), implicating their involvement in re-activation of spliced *FLC* expression and highlighting a role for this previously ignored source of genetic diversity outside of the *FLC* gene body.

For the two lines that did reactivate expression, the magnitude of that reactivation was modest compared to Lov-1 itself (with T30/T10 ratios of 1.1 and 1.3 compared to a ratio of typically about 4 for Lov-1). This aligns with other data collected by other members of the lab: analysis of lines in which the Lov-1 *FLC* allele has been introgressed into a Col-0 (rapidly vernalising) background has implicated the involvement of other regions of the genome, other than *FLC*, in production of the Lov-1 vernalisation phenotype. Thus this result supports the involvement of the +598 SNP in *FLC* reactivation, and highlights the contributions of cis-

variation (namely the +598 SNP in this case) as well as other sources of genetic diversity other than that contained in the *FLC* gene body, to the variation in vernalisation response seen across the accessions.

Additionally, a notable result was the differential spliced and unspliced profiles observed. Although perhaps surprising, it is consistent with the results of Chapter 3, whereupon a similar disparity was observed in the spliced and unspliced profiles of *FLC* during expression analyses of the accessions Lov-1 and Edi-0. Though an explanation for these results remains to be established, one possible explanation could be an increase in splicing/co-transcriptional processing efficiency upon cessation of cold and a return to the warmth. Such a phenomenon would reduce the apparent amount of unspliced transcript, as it would be more rapidly spliced and converted to its spliced form. Whilst this is purely speculative, a precedent does exist in the literature: indeed, the splicing efficiency for a number of genes, including *β-globin*[49] and the circadian clock gene *frequency*[50] has been shown to be highly temperature sensitive, with the transcripts for these genes being differentially spliced according to temperature, which in turn impacts on splicing efficiency (defined in this case as splicing that results in the production of the correct, intended transcript). In the case of *β-globin*, temperature significantly influenced splice site selection, and in turn, impacted the useage of aberrant splice sites that resulted in production of non-sense transcripts.

4.5 Future Experiments.

4.5.1 Generation of Single SNP Constructs: An interesting avenue of exploration would be to generate and analyse constructs carrying Lov-1 and Edi-0 *FLC* alleles in which the only alteration is to the +598 SNP, converting the Lov-1 polymorphism to its Edi-0 equivalent and vice versa. This would allow a more precise establishment of the relationship (if any) between the +598 SNP and the polymorphisms located in the promoter region of *FLC* as well as definitive establishment of the involvement of said SNP in production of the differential vernalisation phenotypes seen between these two accessions.

4.5.2 Identifying Suitable Individual Transgenic Lines: Additionally, previous work carried out in the lab has highlighted significant expression variability in transgenic lines where copies of *FLC* have been transformed in. This apparent high sensitivity to genomic context of insertion likely lies with *FLC*'s possession of a number of distinctive features (including both

a gene loop[51] and an R-loop[52], amongst others) that require a specific genomic context in order to recapitulate expression as it is in non-transgenic backgrounds. Thus, further screening of each of the individual transgenic plants making up each line in order to identify stably and suitably expressed single-copy insertions would be particularly useful, allowing a significant component of noise to be excluded from further analysis. As well, once these lines had been established, carrying out ChIP to assess H3K27me3 and H3K36me3 dynamics in each of these different transgenics, in particular for LEL +598, would be highly informative in definitively elucidating as to whether the reduced H3K27me3 stability identified in Lov-1 is solely a product of the +598 SNP.

4.5.3 Further Exploring CRISPR/Cas9 Technologies: Given the identification of transgenic variability as a potentially serious issue confounding and masking the legitimate biological effects of the SNPs present in each transgenic line, other alternative approaches might be considered worthy of exploration. Indeed, analysis of the region immediately surrounding the +598 SNP, namely the 28bp stretch of nucleotides that contains the SNP and sits between two functionally important B3-domain binding motifs[25], identified 3 protospacer adjacent motifs. These short motifs (NGG) are an essential pre-requisite for Cas9 activity[53], and their presence in this region allows said region to be targeted for Cas9 cleavage, and the subsequent mutagenesis associated with cleavage. To this end, I have produced, using the Golden Gate technique, constructs containing Cas9 driven under the YAO promoter[54] as well as a number of sgRNAs, singly and in pairs, targeting the PAMs present between the two B3 motifs and in the region containing the +598 SNP. Future work will involve transforming these into Edi-0 and screening for late flowerers- our hope is that Cas9-mediated mutagenesis of the region and elucidation of mutants produced by this technique in which the Lov-1 phenotype has been recapitulated will enable further dissection and exploration of the exact way in which the +598 SNP is acting mechanistically.

Chapter 5: *FLC* Silencing Dynamics Across *Arabidopsis* Accessions During Vernalisation.

5.1 Background.

Previous analyses, both in Chapter 3 of this thesis and elsewhere in the literature[46,47] have identified aspects of the vernalisation process that are subject to variation across *Arabidopsis* accessions. This variation is thought to be the principal driver of the distinct cold-responsive phenotypes observed. The way in which these phenotypes are achieved appears to be diverse, with modulations to the dynamic epigenetic reprogramming of the *FLC* locus in the case of Lov-1, compared with variation in the function and structure of the antisense transcript *COOLAIR*, thought to play a role in the initial transcriptional shutdown and subsequent epigenetic reprogramming of *FLC*, in the case of Var2-6.

However, whilst the mechanistic aspects of the diverse vernalisation requirements of the two accessions are comparatively well understood, an analysis of *FLC* expression dynamics during cold exposure across the accessions at high temporal resolution remains incomplete. Additionally, another little explored aspect of the vernalisation process is the effect of temperature on the efficiency of vernalisation. Previous analyses have shown that Lov-1 vernalises more efficiently at 8^{o}c rather than 5^{o}c[55]. Characterisation of the effects of different temperatures on *FLC* silencing and suppression in other accessions had not been undertaken.

To this end, expression of unspliced and spliced *FLC* at fine temporal resolution during the cold was undertaken and assayed using qPCR analysis, across a diversity of accessions (ColFRI, Lov-1 and Var2-6) and at two different temperatures (5^{o}c and 12^{o}c).

5.2 Aim.

- To investigate the dynamics of unspliced and spliced *FLC* expression across *Arabidopsis* accessions during vernalisation at different temperatures.

5.3 Results.

5.3.1 Initial levels of spliced *FLC* prior to cold exposure differed between the accessions:
Analysis of the expression of spliced *FLC* transcripts in each of the three accessions prior to

cold exposure revealed a significant difference (Figure 1A, ANOVA, p = 0.016), with Var2-6 possessing the highest starting expression levels (average = 12.27, n = 3), and ColFRI the lowest (average = 6.64, n = 3), and Lov-1 in-between (average = 8.70, n = 2). Further statistical analysis using Tukey's Honest Significant Difference (TukeyHSD) method revealed that Lov-1 spliced *FLC* levels did not differ significantly from that of Var2-6 (p = 0.1) or ColFRI (p = 0.37), but that expression of spliced *FLC* transcripts in Var2-6 differed significantly to ColFRI (p = 0.014). Thus, there appear to be significant and biologically relevant differences between the accessions in terms of their starting levels of spliced *FLC* prior to cold exposure.

5.3.2 Levels of unspliced *FLC* did not differ between the accessions before cold exposure: Given the apparent significant difference in levels of spliced *FLC* between the accessions, and additionally, in light of the disparity between the spliced and unspliced *FLC* profiles observed in Chapters 3 and 4 of this thesis, expression of unspliced *FLC* was then assayed. In contrast to the analysis of spliced *FLC* levels prior to cold exposure, no significant difference was observed between the three accessions (Figure 1B, ANOVA, p = 0.07), and follow up analysis with TukeyHSD also revealed a lack of statistical significance (Col-Var p = 0.07, Col-Lov p = 0.16, Lov-Var p = 0.93). Thus, as for previous analyses presented in this thesis, comparisons of spliced and unspliced *FLC* profiles prior to cold exposure across the accessions revealed a disparity whereupon differences observed in spliced *FLC* profiles are not observed in those of unspliced *FLC*.

5.3.3 All accessions vernalised more rapidly at 5^{o}c than 12^{o}c: The question was then asked as to whether the efficiency of vernalisation differed for a single accession at the two different temperatures. Statistical analysis revealed that each accession vernalised more rapidly at 5^{o}c (Figure 1C) than 12^{o}c (Figure 1E), indicative of a faster rate of suppression of spliced *FLC.* Comparisons of the absolute values for spliced *FLC* transcript levels for each accession at the two different temperatures revealed statistically significant differences across all three lines (ANOVA, ColFRI p = 0.001, Lov-1 p = <0.001, Var2-6 p = <0.001).

5.3.4 Accessional variation in vernalisation rates were observed for both temperatures analysed: Given the significant differences in vernalisation rate highlighted by comparisons of 5^{o}c and 12^{o}c for individual accessions, rates were then compared across the accessions for a given temperature. However, considering the variation seen in starting levels

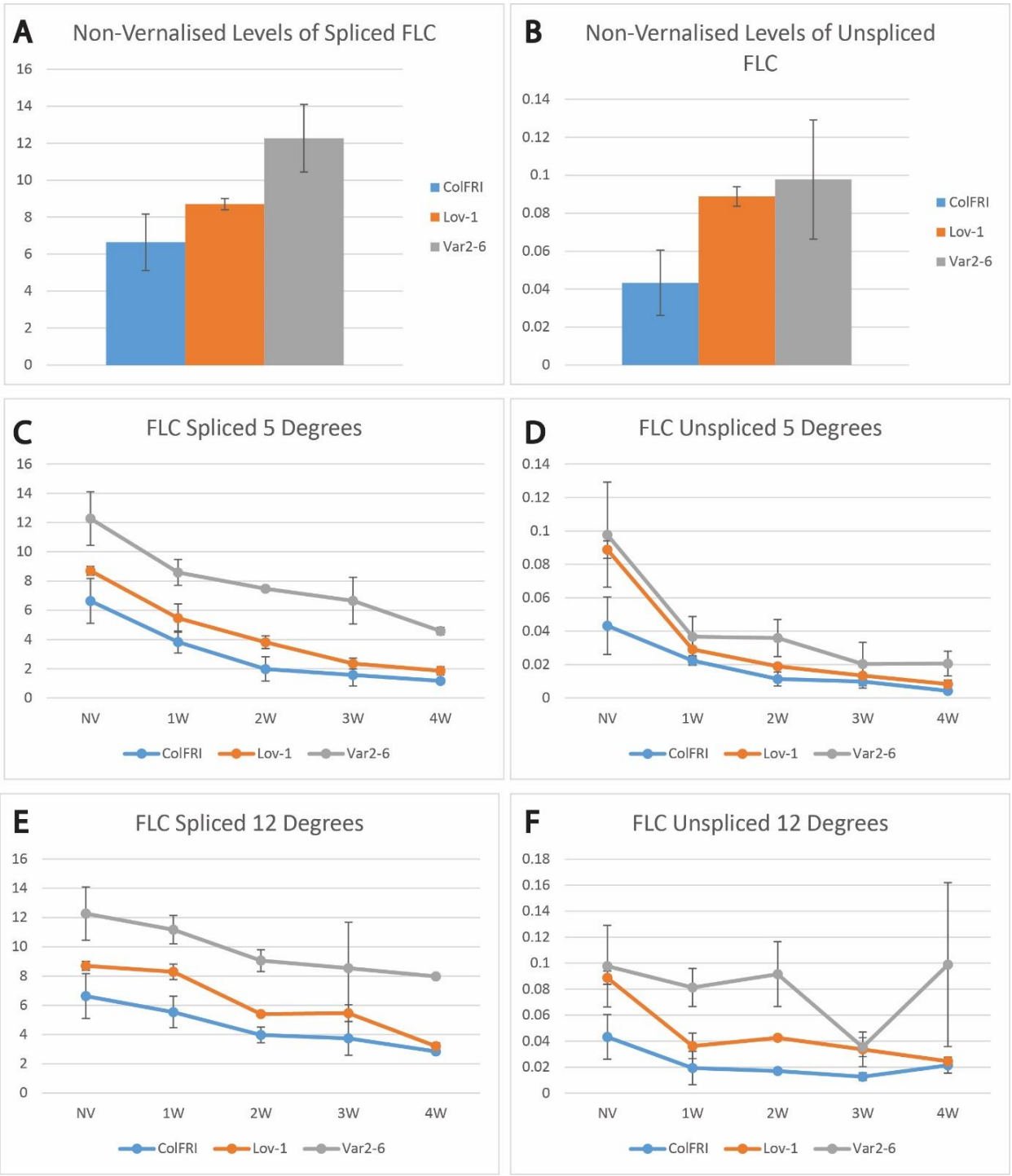

Figure 1: Spliced and unspliced *FLC* expression analysis reveals both accessional and temperature based differences in the cold-induced shutdown of *FLC*.

Plants given either no cold (NV), 1 week (1W), 2 weeks (2W), 3 weeks (3W) or 4 weeks (4W) of cold. Values are mean ± standard error from three biological replicates, and are all normalised to UBC expression. (A) Non-vernalised levels of spliced *FLC* across three accessions. (B) Non-vernalised levels of unspliced *FLC* across three accessions. (C) Analysis of spliced *FLC* expression at 5°c. (D) As above, except that during cold exposure, plants were vernalised at 12°c rather than 5°c. (E) As for (C) except unspliced *FLC* was assayed. (F) As for (D) except unspliced *FLC* was assayed.

of *FLC* observed across the accessions, levels of *FLC* were first normalised to non-vernalised levels. Subsequent statistical analysis revealed that Var2-6 vernalised significantly more slowly than both ColFRI ($p < 0.0001$) and Lov-1 ($p < 0.0001$) at $5^{o}c$. There was no significant difference between the rates of vernalisation observed in Lov-1 and ColFRI ($p = 0.277$). These results therefore highlight accessional differences in the rate of vernalisation, separate to differing initial *FLC* levels, as a potential driver of the distinct vernalisation requirements observed.

Similarly, a statistically significant difference in vernalisation rates was observed at $12^{o}c$, with ColFRI vernalising at a more rapid rate than Var2-6 ($p = 0.018$), in addition to no difference in the vernalisation rates of ColFRI and Lov-1 ($p = 0.235$). In contrast to the results for $5^{o}c$ however, there was no discernible difference between the vernalisation rates of Var2-6 and Lov-1 ($p = 0.164$), indicating differential temperature sensitivities across the accessions with respect to vernalisation.

5.4 Discussion.

Of significant interest from this analysis was the marked difference in initial spliced *FLC* levels observed across the accessions. The higher levels of *FLC* prior to cold exposure observed in Var2-6, compared to Lov-1 and ColFRI, raises the question as to whether this might have adaptive consequences, facilitating the longer vernalisation time required for Var2-6. If so, it would hint at Lov-1 and Var2-6 achieving their slowly vernalising phenotypes through distinct and different mechanisms- whereas Var2-6 appears to produce this through a combination of raised *FLC* levels coupled with a slower rate of vernalisation during the cold, the slowly vernalising phenotype in Lov-1 is mediated through modulation and alteration of the stability of the repressive chromatin mark H3K27me3 following cold exposure upon return to the warmth. Thus, the results presented in this chapter and elsewhere in the thesis point to divergent mechanisms underpinning convergent reproductive strategies across a diversity of *Arabidopsis* accessions.

As with the results presented in Chapter 3 and Chapter 4 of this thesis, a disparity was observed in the unspliced and spliced profiles of *FLC*. This manifested in a number of ways, and was evident at a number of different points during the temporal analysis. Initially, prior to cold exposure, a difference between the two profiles was observed: whereas spliced

levels of *FLC* differed between Lov-1 and Var2-6, such a phenomena is not observed in unspliced levels. Such a disparity points to possible accessional variation in a number of processes, such as splicing efficiency, co-transcriptional processing or mRNA half-life, that would affect conversion of unspliced *FLC* to its spliced form and in doing so account for the disparity observed. Additionally, a similar disparity was observed during cold exposure, whereupon spliced *FLC* levels in Lov-1 and Var2-6 diverged far more substantively than unspliced levels. These results together point to a novel way in which accessional variation in *FLC* dynamics might be achieved, independent of chromatin modulation and alterations to transcription.

Lastly, whilst the observation of slower vernalisation rates at 5^{o}c compared to 12^{o}c across the accessions is in keeping with previous observations reported in the literature[55], the identification in this analysis of accessional differences in the efficiency of vernalisation at different temperatures represents a novel finding. That Var2-6 vernalises more slowly than Lov-1 and ColFRI at 5^{o}c hints at a previously under-explored mechanism by which the slowly vernalising phenotype of that accession might be achieved. Additionally, the fact that this significant difference in vernalisation rate between Var2-6 and Lov-1 is lost at 12^{o}c highlights the existence of differential temperature sensitivity profiles across the accessions, and warrants further investigation.

5.5 Future Experiments.

5.5.1 Investigation of expression patterns of key vernalisation components over the same time period: Given the significant variation observed between accessions in both vernalisation rates and vernalisation efficiency at different temperatures, an interesting avenue to explore in future experiments would be to analyse the expression patterns and functionalities of key components involved in the shutdown of *FLC*, such as *VIN3* and *COOLAIR*. Exploring if any differences exist between accessions would likely prove instructive, and significantly further our knowledge of the molecular events underpinning the diverse vernalisation requirements observed among the accessions.

5.5.2 Analysis of lines representing crosses of ColFRI and other accessions: The lab has generated a number of lines in which the copy of *FLC* from a particular accession has been backcrossed multiple times to ColFRI, producing plants that possess a copy of *FLC* from a

different accession, but in a ColFRI genetic background. Analysis of the dynamics of *FLC* shutdown in these lines, as well as the dynamics and nature of the epigenetic reprogramming of the locus that occurs upon cold exposure, will establish the comparative contributions of variation at the *FLC* locus compared to elsewhere within the genome to differing vernalisation requirements.

Chapter 6- Discussion

This thesis sought to investigate the natural diversity in vernalisation requirements present across *Arabidopsis thaliana*, and to explore the basis for the distinctive cold-responsive phenotypes observed among accessions. Specifically, it sought to better characterise the slowly vernalising accession Lov-1, and to better resolve both the causative genetic variation underpinning its distinct vernalisation requirement, as well as the way in which the process of vernalisation has been altered to produce this specific cold responsive phenotype. This thesis also examined *FLC* silencing dynamics across a wider range of accessions to examine the molecular diversity present between accessions in the way in which they vernalise and suppress *FLC* expression.

Considering these aims, the work contained in this thesis has made a number of significant advancements. The ChIP experiments presented in Chapter 3 have uncovered accessional differences in the dynamic epigenetic reprogramming of the *FLC* locus as a key driver of distinct vernalisation responses among different accessions. And in tandem with the analyses of transgenic lines containing chimeric *FLC* loci in Chapter 4, the causative genetic features underpinning the Lov-1 phenotype have been better resolved. Moreover, the work presented in Chapter 5 highlights differences in the dynamics of *FLC* shutdown during vernalisation in the slowly vernalising accessions Lov-1 and Var2-6, indicating that these accessions have exploited distinct evolutionary pathways to achieve convergent phenotypes. These results also yielded novel insights beyond their immediate scope, most notably highlighting the contributions of variation in post-transcriptional processing across the accessions to altered *FLC* dynamics, and by extension, altered vernalisation requirements.

These results will be discussed in further detail throughout this chapter, and in context of the stated aims introduced at the beginning of this thesis. Specifically, their implications for our understanding of both vernalisation and, more broadly, evolution, will be considered. They will be reconciled in light of previous work and then finally, future avenues of exploration and experimentation suggested.

6.1 Explore Histone Dynamics In Lov-1 And The Rapidly Vernalising Edi-0.

The work of Coustham et al, 2012[46] had identified altered dynamics in the chromatin profiles of the slowly vernalising accession Lov-1, and the rapidly vernalising ColFRI, as a

driver of the differential vernalisation requirements observed in these accessions. ChIP experiments described in this thesis, analysing the dynamics of histone modifications in Lov-1 and the naturally occurring rapid vernaliser Edi-0, confirm altered chromatin dynamics as a feature of the distinctive vernalisation phenotypes observed. However, in contrast to ColFRI, where the rapidly vernalising phenotype is a product of increased levels of H3K27me3 prior to vernalisation, in Edi-0, this appears to be due to enhanced stability of the gene body H3K27me3 modifications upon return to the warmth. Thus, exploration of histone dynamics across these two accessions has significantly furthered our understanding of the exact way in which the dynamic epigenetic reprogramming of the *FLC* locus that occurs upon cold exposure varies between accessions. As such, this work shows that altered epigenetic silencing dynamics are a driver of divergent reproductive strategies across *Arabidopsis thaliana* accessions.

Interestingly however, this does not appear to be the sole way in which the process of vernalisation can be modulated in order to achieve differential vernalisation requirements. Analyses presented in Chapter 5, as well as from existing literature[47], indicate that the slowly vernalising Var2-6 achieves its phenotype through a distinct pathway from that of Lov-1. Whereas the slowly vernalising phenotype in Lov-1 appears to be a product of modulations to the stability of chromatin modifications following vernalisation, in Var2-6, slow vernalisation appears to be a product of high starting levels of *FLC* in concert with a decreased rate of silencing during cold exposure. This is in agreement with the recent identification of altered antisense splicing as a cause of Var2-6's phenotype[47], given *COOLAIR*'s involvement in both setting basal levels of *FLC*[22] as well as the initial transcriptional shutdown that occurs upon cold exposure[56]. Thus, in addition to better resolving the mechanism underpinning the Lov-1 phenotype, this work also presents evidence for divergent evolutionary routes to convergent adaptive phenotypes, and supports the idea that different aspects of vernalisation appear to have been altered by different accessions to achieve the same phenotype. Further work exploring the epigenetic diversity across other accessions, including Var2-6, using ChIP, would prove highly instructive in assessing how common the phenomenon seen in Lov-1 is. Additionally, it would further elucidate the interplay between the variation in mechanistic features identified in Var2-6 and the dynamics of chromatin modifications at *FLC* in this accession.

6.2 Assess The Importance Of The +598 SNP And Its Contribution To The Lov-1 Slowly Vernalising Phenotype.

In addition to better characterising the variation in chromatin dynamics observed in Lov-1, this thesis also sought to build upon the work of Coustham et al in another way, and further resolve the causative genetic variation underpinning the Lov-1 phenotype. The work presented in Chapter 4, with supporting evidence from Chapter 3, has pinpointed the contribution of the +598 SNP in generating the distinctive vernalisation requirement observed in Lov-1. However, this work also highlighted the contribution of sources distinct from the region containing the 4 SNPs. That the magnitude of *FLC* reactivation observed in the domain swap lines was lower than that observed in Lov-1 itself points to the role of other sources of genetic variation in contributing to its phenotype. And indeed, a close consideration of expression in these lines highlighted the possible contribution of hitherto unconsidered diversity in the promoter region of *FLC* to the Lov-1 vernalisation phenotype. Additionally, given that the domain swap lines represented transgenic lines in a ColFRI background, the possibility of contributions from variation at other loci other than *FLC* cannot be ruled out. Thus, future experimentation should focus on dissecting the complex roles of different types of variation in production of the vernalisation requirement observed in Lov-1.

Additionally, identification of the factors that interact with the genetic variation at *FLC* remains both a priority and an area in which little progress has been made. However, recent modelling efforts in conjunction with the Howard group at the John Innes Centre have implicated altered functional efficiency of the H3K27me3 demethylase ELF6[57] in contributing to the Lov-1 phenotype, distinguishing it as an avenue worthy of further exploration.

6.3 Investigate *FLC* Silencing Dynamics In ColFRI, Lov-1 And Var2-6.

Investigations of *FLC* silencing dynamics across a variety of accessions revealed a number of key differences that manifested at different stages of the vernalisation process. In keeping with the data presented in Chapter 3, no differences in *FLC* expression were detected between Lov-1 and the rapidly vernalising ColFRI during vernalisation itself, supporting the idea that it is events following cold exposure, upon the return to warmth, that act to

differentiate the two in terms of vernalisation requirement. Additionally, the observation of high starting *FLC* levels and a slower rate of vernalisation in Var2-6 is congruent with the recent report of altered *COOLAIR* dynamics and functionality in this accession, given the role of this collection of antisense transcripts in both of these processes. Thus, the analysis of *FLC* dynamics presented in Chapter 5 corroborates other results presented in this thesis, as well as those in the literature.

Additionally, analysis of *FLC* expression across the accessions during cold exposure highlighted novel contributions from co-transcriptional processes including RNA splicing. This arose from the observation of a disparity between spliced and unspliced *FLC* profiles, a phenomenon seen throughout the results presented in this thesis, but most notably observed in Chapter 5. Such a disparity highlights the contribution of diversity between accessions in processes centred around co-transcriptional RNA processing as a contributing factor to the diversity in vernalisation requirements observed. Thus, this work points towards the role of a number of different molecular events, ranging from splicing to co-transcriptional processing to RNA decay, in contributing to the distinct vernalisation requirements possessed by the analysed accessions. Whereas work surrounding accessional differences in splicing, are comparatively well explored in the literature, at both the single loci level[58] and genome wide[59], diversity across accessions in other co and post-transcriptional processes remains poorly explored; thus, the findings from this thesis represent a potentially novel avenue for further exploration.

6.4 Conclusion.

Study of the diversity in the nature of vernalisation across natural populations of *Arabidopsis thaliana* has informed our understanding of the ways in which organisms can adapt to changing environments. Specifically, it has enabled elucidation of the ways in which epigenetic phenomena can be shaped by evolutionary forces, and has provided a detailed understanding of the ways in which selection can act on specific aspects of complex developmental processes at the single gene level.

This thesis has presented a close study of the molecular mechanisms that underpin the variation in vernalisation requirements across a diversity of *Arabidopsis thaliana* accessions. In doing so, it has sought to further our knowledge of natural diversity in vernalisation

requirements in three ways: the causative genetic features underpinning the variation; how these features modulate the process of vernalisation; and, finally, the effect this modulation has on dynamics of *FLC* suppression during cold exposure.

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