Vernalisation in *Arabidopsis thaliana*: Investigating how *COOLAIR* is induced in response to cold temperature

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Abstract

Arabidopsis thaliana, a winter annual, requires a period of prolonged cold to permit flowering, in a process known as vernalisation. Vernalisation results in the transcriptional shutdown and epigenetic silencing of a locus encoding the floral repressor, FLOWERING LOCUS C (FLC). Relatively little is known about the thermosensors involved in relaying a prolonged cold temperature signal into silencing of *FLC,* however it is known that *COOLAIR*, a set of antisense long non-coding RNAs (lncRNAs), and VIN3 are both transcriptionally upregulated in response to weeks of cold.

The aim of my work described in this thesis was two-fold, to firstly identify the potential factors involved in *COOLAIR* induction and secondly to investigate the thermosensory inputs to *COOLAIR* induction, in an effort to identify how prolonged cold temperature is being sensed at *COOLAIR.*

In summary, a non-biased forward genetic screen mutant analysis identified candidate thermosensors required for the cold induction of *COOLAIR*, including ARP6, a core component of a histone remodelling complex. A complementary reverse genetic approach, formulated based on *cis* sequence conservation in the *COOLAIR* promotor, identified known cold affected redundant factors that may activate *COOLAIR* expression in response to cold temperature. I also carefully designed temperature regimes to more accurately represent fluctuating field temperatures, to ascertain the temperature inputs being detected at *COOLAIR*. Experiments tracking *COOLAIR* expression under different temperature regimes isolated likely distinct temperature inputs at COOLAIR contributing to a distributive view of thermosensing. This work has provided much scope for further investigations that could ultimately lead to the characterisation of thermosensing during vernalisation.

Contents

List of Figures

1 Introduction

3 Identify regulators of cold-induced COOLAIR expression

4 Dissect COOLAIR induction in the identified mutants

5 Investigate COOLAIR induction in response to different temperature regimes

8 Appendix

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1 Introduction

The goal of my research was to elucidate the thermosensory inputs and identify candidate factors that are involved in the induction of a set of lncRNAs, collectively named *COOLAIR*, in response to cold temperature in *Arabidopsis thaliana. COOLAIR* is transcribed antisense to *FLC,* a pivotal floral repressor. Induction of *COOLAIR* in response to prolonged cold temperature influences downregulation of FLC, alongside other factors resulting in a process termed vernalisation. Vernalisation is the induction of flowering in response to exposure to prolonged cold temperature or, in other words, winter. At the beginning of the project it was not known how *COOLAIR* expression is induced by cold temperature or even what temperature inputs affect *COOLAIR* expression.

1.1 Temperature affects everything

World-renowned physicist Richard Feynman described temperature as 'the jiggling of atoms'. Temperature affects the structure of molecules, the rate of chemical and enzymatic reactions, and the probability a reaction happens at all (Gibb's free energy). Cells are biochemical engines driven by highly structured molecules, so cells and whole organisms need to sense and respond to temperature or risk loss of cell homeostasis.

Moreover, some organisms rely on temperature to inform their developmental decisions. *Arabidopsis* plants exposed to high temperature have an elongated hypocotyl and flower early (Gray & Estelle, 1998; Kumar & Wigge, 2010). Additionally, some plants will not flower until experiencing a prolonged period of cold, a process called vernalisation (Simpson & Dean, 2002).

Temperature responses often result in clear phenotypes such as the length of a hypocotyl, the timing of flowering or the RNA expression profiles of cold-acclimatised plants (Jia et al., 2016; Zhao et al., 2016). On the other hand, temperature sensors, which can detect a temperature signal and relay this to cause a response, have proven somewhat more difficult to elucidate (Knight & Knight, 2012). Given temperature broadly affects biochemistry, finding the causative change in a factor required for a temperature response is akin to finding a needle in a haystack. Additionally, recent research modelling temperature inputs to vernalisation has revealed the need for multiple thermosensors, akin to finding multiple needles in a haystack (Antoniou-Kourounioti et al., 2018).

1.2 Known Thermosensing Pathways in Arabidopsis

Temperature responsive phenotypes and pathways downstream of the temperature sensing event are well documented in *Arabidopsis* however the initial thermosensing events are less understood. Below two temperature responsive events; altered plant development including early flowering and elongated hypocotyl growth in response to high temperature and cold acclimation of plants in response to low temperatures will be examined.

In response to 27°C growth temperature compared to 22°C, *Arabidopsis* plants transition between developmental stages more rapidly resulting in plants that have extended hypocotyls and flower early (Kumar & Wigge, 2010). Mutant analysis has revealed the role of transcription factors and histone remodellers in causing these developmental changes.

Phytochrome Interacting Factor 4 (PIF4), a basic helix turn helix transcription factor, is required for hypocotyl elongation and is required and sufficient for early flowering in response to high temperature (Koini et al., 2009; Kumar et al., 2012). *PIF4* expression at 22°C peaks just before dawn and this peak is increased two-fold in response to 27°C. Modelling experiments supported by expression data have implicated the temperature responsive binding of Early Flowering 3 (ELF3), a transcriptional repressor, to the promotor of *PIF4* in this phenomenon (Box et al., 2015). PIF4 activity is known to be repressed by phytochromes through protein degradation (Lorrain & Fankhauser, 2008). High temperature has recently been shown to increase the rate phytochromes are transformed back to their inactive state meaning that thermosensing by phytochromes directly impacts PIF4 activity (Jung et al., 2016; Legris et al., 2016).

arp6-10 Arabidopsis mutants phenotypically resemble plants grown at 27°C, with an elongated hypocotyl and early flowering, despite being grown at 12°C (Kumar & Wigge, 2010). Actin- related protein 6 (ARP6) is part of the Swi2/Snf2 – related 1 (SWR1) histone remodelling complex and is required for H2A.Z deposition throughout chromatin (Deal & Meagher, 2007). H2A.Z removal from gene promotors has been implicated expression of the 'warm' transcriptome, which is why in *arp6-10* plants, where there is very little H2A.Z, the 'warm' transcriptome is expressed and plant resemble plants grown at 27°C. It is not yet clear if H2A.Z nucleosomes respond directly to increased temperature or if other factors respond and mediate H2A.Z removal.

Overall then, thermomorphogensis in *Arabidopsis* is controlled in part by the activity of PIF4 and the removal of H2A.Z that both lead to expression of a 'warm' transcriptome. Altered protein binding (ELF3 and phytochrome B) as a result of temperature mediated transitions between active and inactive states is a thermosensing mechanism used in this process. H2A.Z containing nucleosomes may also themselves directly respond to temperature, but this could happen through indirect means.

Arabidopsis plants also respond to cooler temperatures. *Arabidopsis* plants exposed to low temperatures ~5°C and then to freezing conditions are more freezing tolerant than those plants exposed to freezing conditions without prior exposure to 5°C. This cold acclimation process is associated with expression of cold-regulated (COR) genes (Gilmour, Hajela, & Thomashow, 1988). Discovery of a cis-acting regulatory sequence in the promotors of a group of COR genes (Yamaguchi-Shinozaki & Shinozaki, 1994) led to the isolation of three C – Repeat/ Dehydration response element binding factors (CBFs) (Gilmour et al., 1998; Stockinger, Gilmour, & Thomashow, 1997). CBF1, 2 and 3 are transcriptionally induced in response to hours at 5°C and then in a partially redundant manner induce expression of a suite of COR genes initiating the cold acclimation transcriptome (Gilmour, Fowler, & Thomashow, 2004; Jia et al., 2016; Zhao et al., 2016).

CBF1,2 and 3 are regulated by multiple transcription factors, including positive and negative regulators, that bind cis elements in the CBF gene promotors. Inducer of CBF expression (ICE1) is a positive regulator of the CBFs and in response to cold temperature ICE1 protein is stabilized as a result of sumolyation and repression of ubiquitination (Chinnusamy et al., 2003; C. H. Dong, Agarwal, Zhang, Xie, & Zhu, 2006). Other positive regulators of the CBFs include Calmodulin binding transcriptional activator 3 (CAMTA3) (Doherty, Van Buskirk, Myers, & Thomashow, 2009) and circadian regulators Circadian clock associated 1 (CCA1) and Late Elongated Hypocotyl (LHY) (M. A. Dong, Farre, & Thomashow, 2011). Negative regulators include MYB domain protein 15 (MYB15) (Agarwal et al., 2006) and PIF4 (Jiang et al., 2017; Lee & Thomashow, 2012).

Interestingly, expression of CBF1, 2 and 3 is not regulated in the same way. CBF2 is a known negative regulator of CBF1 and CBF3 and in *cbf2* plants freezing tolerance after cold acclimation is increased coincident with higher and sustained expression of CBF1, CBF3 and COR genes (Novillo & Salinas, 2004). Additionally, CAMTA3 and 5 are required for cold inducible expression of CBF1 and CBF2 in response to cold treatments in the day or night whilst other factors such as CCA1 and LHY are

required for cold induction of CBF2 and CBF3 in response to cold treatments during the day (Kidokoro et al., 2017).

The actual temperature sensing events that occur in response to cold temperature resulting in cold acclimation are less well understood relative to the response itself. The positive and negative transcriptional regulators previously mentioned are somehow influenced by temperature to control CBF expression. PIF4 may be regulated as previously described by ELF3 and PHYB (Box et al., 2015; Jung et al., 2016; Legris et al., 2016) In this case in warm temperatures PIF4 is expressed more highly and the CBFs are repressed.

Thermosensors responding to cold temperature have proved more elusive. Certain events are known to occur in response to cold temperature including increased cytosolic Ca^{2+} levels, altered membrane fluidity and cytoskeletal rearrangements.

An increase in cytoplasmic Ca^{2+} is known to occur as one of the earliest steps in cold acclimation and is important for the cold acclimation response (H. Knight, Trewavas, & Knight, 1996; M. R. Knight, Campbell, Smith, & Trewavas, 1991; Monroy, Sarhan, & Dhindsa, 1993). The Ca^{2+} signature produced in response to cold temperature depends on the rate of cooling as opposed to the absolute temperature reached, though cooling 10°C over an hour does not produce a response (Plieth, Hansen, Knight, & Knight, 1999). At already low temperatures the system is more sensitive; further drops in temperature cause increased Ca^{2+} elevations. Repeated exposures to drops in temperature attenuate the response producing a Ca^{2+} signature linked to memory of cold (H. Knight et al., 1996). An increase in Ca^{2+} likely acts as a secondary messenger within the cell causing modification of positive regulators of CBF expression. CAMTA3 is a known calmodulin binding protein and its activity will therefore likely be impacted by the presence or absence of Ca^{2+}.

It has been hypothesised that these Ca^{2+} elevations could happen as a result of temperature induced changes in Ca^{2+} channels and recently two Ca^{2+} permeable mechanosensitive channels have been characterised (Mori et al., 2018). Membrane fluidity and the status of the cytoskeleton have also been linked to cold dependent elevation of Ca^{2+}. When the membrane is fluidised, using chemicals, at low temperature, the expected Ca^{2+} influx and cold acclimation doesn't occur and if the membrane is rigidified, using chemicals, at 25°C, Ca^{2+} increases and cold acclimation occurs despite the absence of cold temperature (Orvar & Dhindsa, 2000). Chemicals stabilising the cytoskeleton

lead to loss of cold-dependent Ca $^{2+}$ elevation whereas chemicals that destabilise microfilaments lead to a Ca^{2+} influx regardless of temperature (Mazars et al., 1997).

Overall, the positive and negative regulators of CBF induction, which is important for cold acclimation in Arabidopsis, are well studied, however the thermosensors involved in this process are not well understood. Ca^{2+} signatures that occur in response to drops in temperature are likely to be important for sensing cold temperatures though it is unclear how drops in temperature cause these Ca^{2+} elevations.

1.3 Deciding to flower and the role of temperature

Flowering is a critical developmental decision that ultimately results in the propagation of genetic information to the following generation. The decision to flower is determined by the expression of floral regulators particularly FLOWERING LOCUS T (FT). FT is expressed in the leaves and transported to the shoot apical meristem, via the phloem, to initiate floral organ development (Wigge, 2011). FT expression is intricately controlled by multiple inputs including day length, age, temperature and the level of FLC, a transcriptional repressor of FT. FLC itself is regulated by the activating FRIGIDA pathway and the repressive autonomous pathway whilst being specifically repressed in response to prolonged period of cold, a process known as vernalisation.

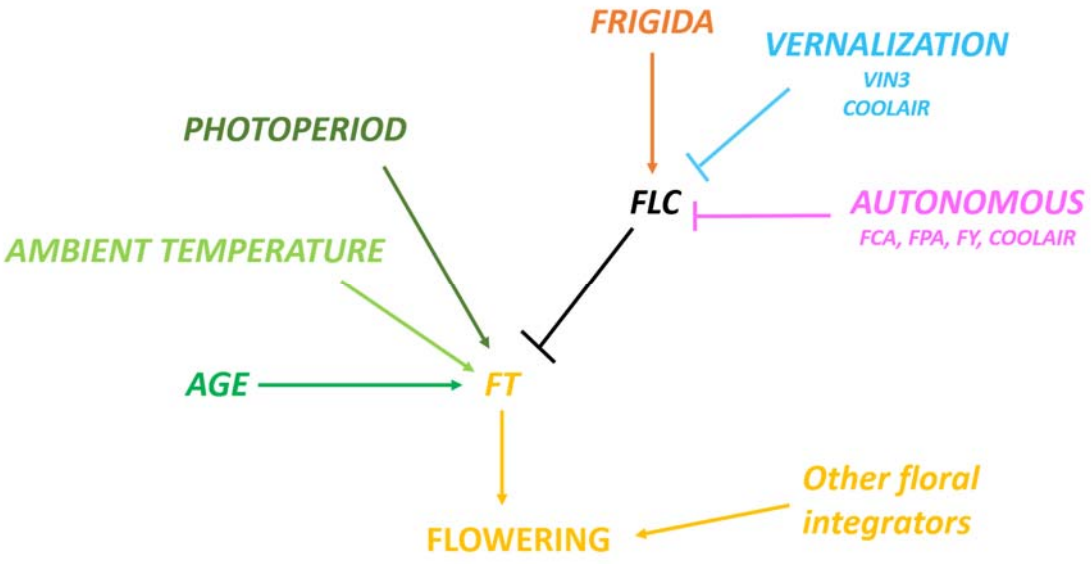


Figure 1 – 1 Multiple Inputs influence FT expression
FT, alongside other floral integrators, controls flowering time in Arabidopsis. FT expression is influenced by age, ambient temperature, photoperiod and the level of FLC which itself is influenced by the activating FRIGIDA pathway and repressive autonomous and cold-specific vernalisation pathways.

Elevated temperatures have been shown to both activate (Sureshkumar, Dent, Seleznev, Tasset, & Balasubramanian, 2016) and repress flowering (Bouche, Detry, & Perilleux, 2015) in a context-dependent manner, whilst prolonged cold temperatures may be required to permit flowering, in a process termed vernalisation (Simpson & Dean, 2002). Requiring vernalisation ensures flowering occurs in spring rather than autumn to maximise reproductive success. The process of vernalisation will form the basis of this thesis and is explained further below.

1.4 Vernalisation: The silencing of *FLC* in response to prolonged cold

FLC, a MADS box transcription factor, is a repressor of FT and therefore a repressor of flowering. FT expression cannot reach critical levels to initiate flowering when *FLC* is expressed to a high level.

Exposure of seedlings to prolonged cold, vernalisation, results in the silencing of *FLC* (Searle et al., 2006) and the subsequent expression of *FT* and floral initiation. *FLC* levels are determined in the warm by the antagonistic actions of the activating FRIGIDA complex and the repressive autonomous pathway (Figure 1-1). In response to vernalisation, *FLC* is quantitatively silenced; longer periods of cold lead to further downregulation of *FLC*. This is the result of digitally switching off more *FLC* loci over time spent in the cold (Angel et al., 2015; Berry, Hartley, Olsson, Dean, & Howard, 2015). *FLC* silencing can be broadly split into two phases that happen during cold exposure (Figure 1-2). The first phase is transcriptional shutdown of *FLC* and the second phase is nucleation of epigenetic silencing marks, at a region found in intron 1 of *FLC*, by the PRC2 complex. On return to warm, these silencing marks are spread from the nucleation region throughout the rest of the gene body (Angel, Song, Dean, & Howard, 2011; Bastow et al., 2004; Berry & Dean, 2015) resulting in the stable silencing of *FLC* and therefore a loss of repression at *FT.*

Given this thesis focuses on the elucidation of thermosensors in vernalisation only the two phases that occur during prolonged cold, the transcriptional shutdown of *FLC* and the nucleation of silencing marks, will be detailed below.

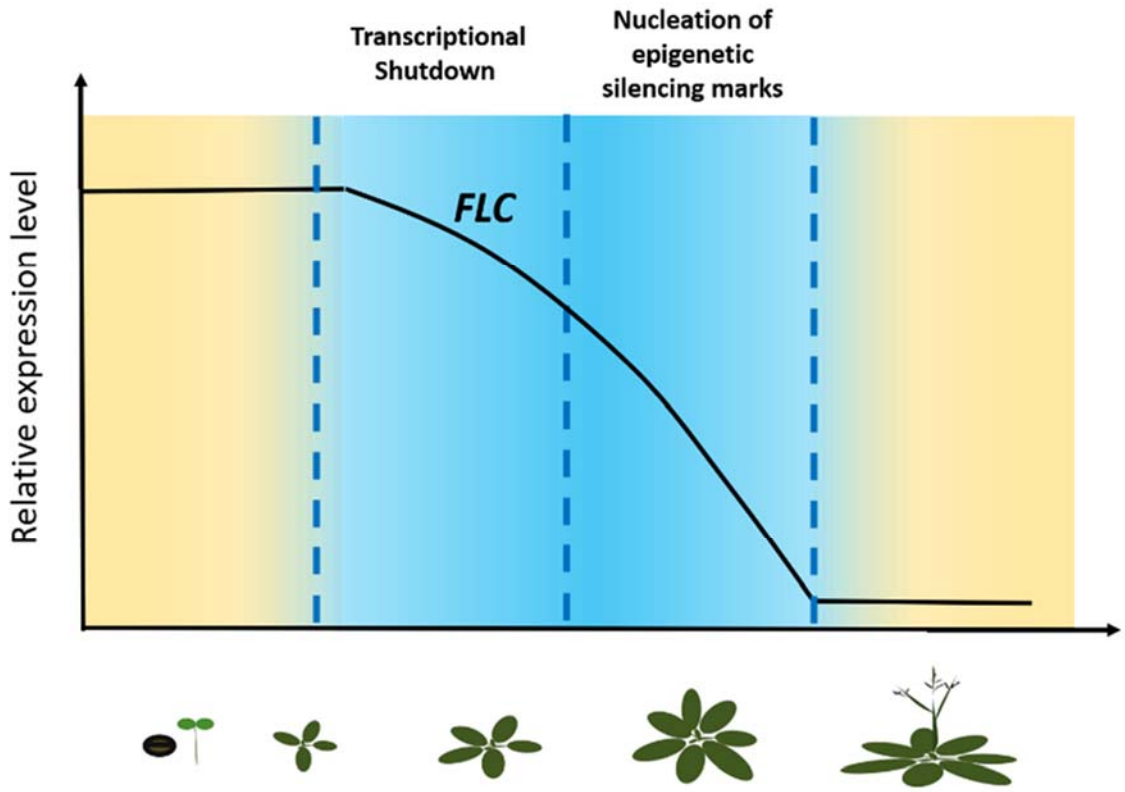


Figure 1 -2 - *FLC* is transcriptionally downregulated in response to cold to permit flowering
FLC levels are relatively highly expressed in seedlings but this decreases if seedlings are moved into prolonged cold temperatures due to the transcriptional shutdown of *FLC* and the nucleation of epigenetic silencing marks. On return to warm, *FLC* levels remain low despite the loss of the cold temperature signal and plants are able to flower.

1.5 Silencing of *FLC* in response to cold- Phase 1: Transcriptional Shutdown

Transcriptional shutdown of *FLC* happens in the first two weeks of cold and although it is unclear how this occurs, a series of events are known to happen: increased binding of VP1/ABI3 – like 1 (VAL1) to the nucleation region, the breakage of a gene loop and induction of a set of antisense long non-coding RNAs (lncRNAs) termed *COOLAIR* (Crevillen, Sonmez, Wu, & Dean, 2013; Questa & Dean, 2016; Swiezewski, Liu, Magusin, & Dean, 2009).

VAL1, a DNA-binding protein, recognises cis sequences found within the nucleation region of *FLC* (Figure 1-2) and recruits a histone deacetylase HDA19, reducing *FLC* expression (Questa et al., 2016; Whittaker & Dean, 2017). VAL1 appears to function independently from the breakage of the gene loop and induction of *COOLAIR*.

Prior to cold exposure, there is a gene loop between the promotor of *FLC* and a region 3' of the *FLC* termination site, which is the promotor of a set of alternatively spliced, polyadenylated, antisense,

long non-coding transcripts called *COOLAIR* (Figure 1-3, Figure 1-4B). In the warm, total *COOLAIR* (as measured by primers in the first exon common to all forms) and *FLC* expression levels correlate on a whole plant level, meaning that in a mutant background that increases *FLC* expression relative to WT, total *COOLAIR* expression also increases (Figure 1-4A). *FLC* and distal *COOLAIR* are not however transcribed from the same locus at a given time (Rosa, Duncan, & Dean, 2016; Swiezewski et al., 2009). In response to two weeks of cold, there is breakage of the gene loop, downregulation of *FLC* and induction of all forms of *COOLAIR,* though predominantly proximal and unspliced forms (Crevillen et al., 2013). In response to cold then, *FLC* and total *COOLAIR* levels on a whole plant level anti-correlate relative to WT in the warm (Figure 1-4A). Once broken the gene loop does not reform even on return to warmer temperatures.

Measuring the transcriptional downregulation of FLC in the early stages of prolonged cold can be difficult given the long half-life of the spliced FLC transcript so unspliced FLC levels can be used as a transcriptional readout of the locus during this early phase of downregulation (Csorba et al., 2014).

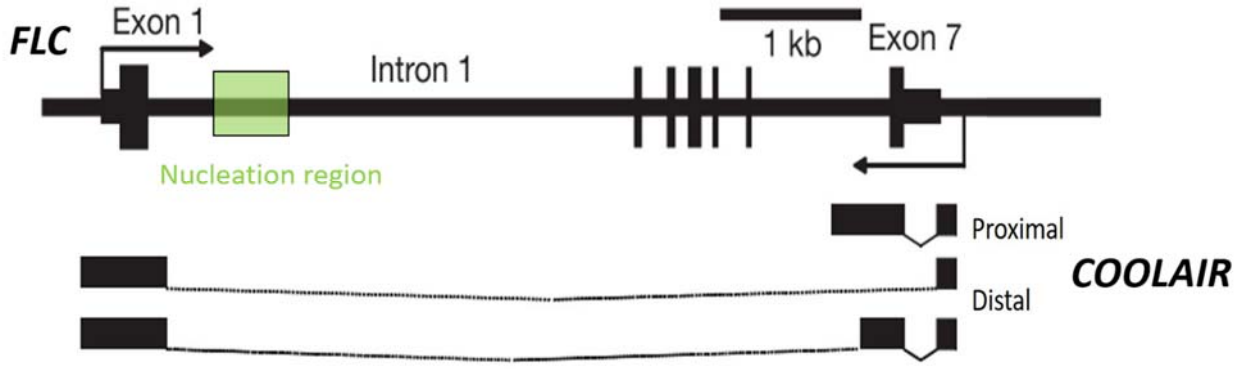


Figure 1-3 – The *FLC* locus showing *COOLAIR* transcripts and the nucleation region. *COOLAIR* transcripts are transcribed downstream of the *FLC* 3' termination site across the whole *FLC* locus*.* *FLC* is made up of seven exons separated by introns. Intron 1 is particularly large and includes the nucleation site, where VAL1 binds and epigenetic silencing marks are added. *COOLAIR* is a set of alternatively spliced polyadenylated transcripts that are categorised into proximal and distal forms.

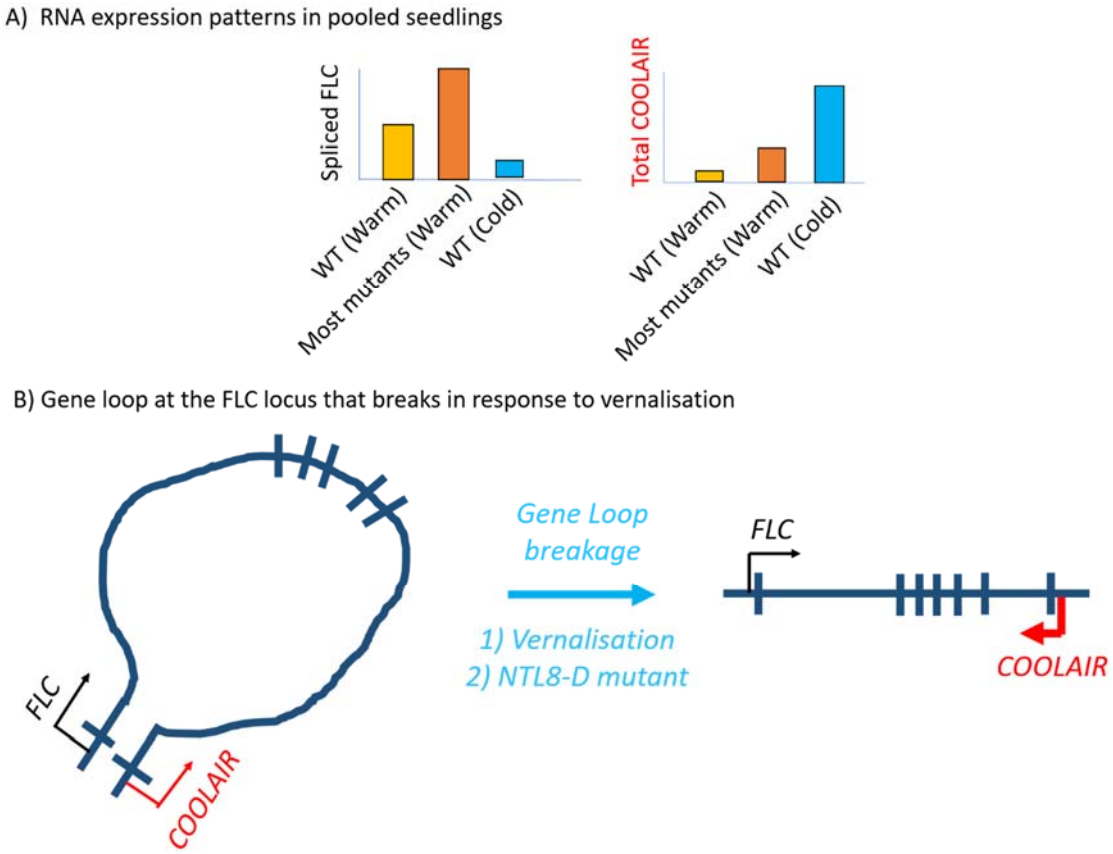


Figure 1-4 – Correlation of *FLC* and *COOLAIR* transcripts and the gene loop at *FLC*
A) Hypothetical expression data from multiple Arabidopsis seedling reveals that *FLC* and *COOLAIR* levels correlate on a whole plant level. In other words, in mutants where *FLC* is increased, *COOLAIR* is also increased. In response to cold, *FLC* and *COOLAIR* levels no longer correlate as *COOLAIR* is increased and *FLC* is decreased. This also happens in an *NTL8* dominant mutant. B) A gene loop exists between the *FLC* and *COOLAIR* promotors. This is broken in response to vernalisation or in the *NTL8* dominant mutant, the only two cases were *FLC* and *COOLAI*R levels do not correlate.

1.6 How does *COOLAIR* induction downregulate *FLC?*

In response to two weeks of prolonged cold, levels of unspliced and proximal *COOLAIR* increase. Analysis of transcription and decay rates in *Arabidopsis* seedlings grown over a range of ambient temperatures showed temperature effects were generally caused by transcriptional changes (Sidaway-Lee, Costa, Rand, Finkenstadt, & Penfield, 2014). Thus, the increase in total *COOLAIR* steady state RNA levels, in response to cold temperature, is presumably through cold promotion of transcription. This is supported by the cold – dependent induction of antisense GFP transcripts in a *COOLAIR* promotor 3' end GFP fusion. In these lines, sense GFP transcripts reduced in level in response to cold, hinting at a direct role of antisense transcripts in *FLC* transcriptional downregulation (Swiezewski et al., 2009).

Complete knockdown of antisense transcripts has proven difficult to engineer without affecting stability of *FLC* sense transcripts (Csorba & Dean, 2014) or upregulating antisense lncRNAs with alternative start sites (Zhu, unpub), however lines with reduced levels of total *COOLAIR* lose active chromatin marks more slowly (Csorba et al., 2014). These active chromatin marks antagonise the nucleation of epigenetic silencing marks so reduced total *COOLAIR* induction increases the time of cold exposure required to fully silence *FLC*. Increased transcription of *COOLAIR* may recruit a, as yet unidentified, H3K36 demethylase or may transcriptionally shutdown FLC expression, resulting in the loss of active chromatin marks.

Single-molecule RNA Fluorescence In – Situ Hybridization (FISH), using probes within distal *COOLAIR* intron 2, revealed that after two weeks of cold exposure, unspliced *COOLAIR* 'clouds' form at *FLC* loci, as a result of transcriptional induction and increased retention at the locus (Rosa et al., 2016). This could lead to the formation of a nuclear compartment with high concentrations of recruited factors. Alternatively, *COOLAIR* could mediate nuclear architecture changes, ultimately resulting in the co-localisation of silenced *FLC* loci (Rosa et al., 2013).

Transcription of proximal and distal *COOLAIR* leads to the formation of co-transcriptional R – loops. R – loops are structures made up of a stretch of ssDNA that has been displaced from dsDNA as a result of the formation of a DNA-RNA hybrid (Drolet et al., 1995; Thomas, White, & Davis, 1976). Nodulin Homeobox (NDX), an ssDNA binding protein thought to stabilise R – loops, affects total *COOLAIR* and *FLC* expression when mutated (Sun & Dean, 2013). As yet, it is not known how R – loop dynamics alter in the cold.

1.7 COOLAIR has a complex relationship with *FLC*

Investigations into the role of *COOLAIR* transcripts, induced by prolonged cold, has further been complicated by the intimate link between *COOLAIR* and *FLC* prior to cold exposure. This link manifests itself in two ways: as a correlation between *FLC* and total *COOLAIR* expression on a whole plant level and as *COOLAIR* being directly required by the autonomous pathway to repress *FLC* expression in the warm.

At a single locus, distal *COOLAIR* and *FLC* are transcribed mutually exclusively and yet on a whole plant level, in mutant backgrounds where FLC or total COOLAIR expression is altered, *FLC* and total *COOLAIR* levels generally correlate. This is to say that in a mutant where FLC levels are increased,

total COOLAIR levels are also increased and vice versa (Figure 1-4). It is therefore very difficult to dissect whether a genotype or mutation primarily affects *FLC* or total *COOLAIR* expression, before this effect secondarily affects sense and antisense expression levels at the locus. *COOLAIR* reporter lines, such as *COOLAIR*: LUC described later in this thesis, that do not express *FLC*, and are therefore independent of *FLC* expression, can be used to dissect this problem (Marquardt et al., 2014; Sun et al., 2013).

FLC and total *COOLAIR* levels have been found to anticorrelate on a whole plant level in just two cases: in response to prolonged cold and in a dominant NAC with transmembrane motif 1 – like 8 (*C*) mutant (Zhao, Nielsen and Dean, unpub) NTL8 is a membrane associated transcription factor that is influenced by salt stress (Kim, Kim, & Park, 2007; Kim, & Park, 2008; Kim & Park, 2007) Interestingly, these two cases are also the only known cases of gene loop breakage between *FLC* and *COOLAIR*.

COOLAIR is required for the autonomous pathway to repress *FLC* prior to cold exposure. The autonomous pathway is vital for setting up the correct *FLC* levels before vernalisation, which is in turn important for adaptation of different accessions to different environments (Duncan et al., 2015). Proximal *COOLAIR* is promoted relative to distal *COOLAIR* in the warm by autonomous pathway components FCA, FY and FPA. FCA, FY and FPA achieve this by promoting efficient termination of RNA Polymerase II transcription at the proximal site. This requires CstF64 and CstF77, for efficient polyadenylation and PRP8 for efficient splicing (Liu, Marquardt, Lister, Swiezewski, & Dean, 2010; Marquardt et al., 2014). Promotion of proximal *COOLAIR* leads to H3K4 demethylation by FLD, also an autonomous pathway component (Figure 1-5) (Wu et al., 2016). In autonomous pathway mutants, distal *COOLAIR* is preferentially made over proximal, gene body H3K4me is high and *FLC* transcription initiation and elongation are faster and more efficient (Wu et al., 2016). *COOLAIR* transcripts are therefore directly involved in FLC repression, through the autonomous pathway.

1.8 Silencing of *FLC* in response to cold- Phase 2: Nucleation of epigenetic silencing marks

FLC is silenced in response to prolonged cold and remains silenced on return to warm despite the loss of the 'cold' environmental signal. This phenomenon implicated epigenetic memory in

vernalisation. Mutants identified from vernalisation screens identified the Polycomb Repressive Complex (PRC2) and accessory proteins as playing major roles in *FLC* silencing.

FLC silencing requires PRC2 components including VRN2, SWN, VRN5 and VIN3 (Chandler, Wilson, & Dean, 1996). Before cold, PRC2 complexes consisting of the core proteins SWN, VRN2, FIE and MSI1 bind dynamically over the entire *FLC* locus (Bond, Dennis, Pogson, & Finnegan, 2009; De Lucia, Crevillen, Jones, Greb, & Dean, 2008; Greb et al., 2007). During the cold, VIN3 is expressed and, with constitutively expressed VRN5, associates with the PRC2 complex, increasing H3K27 methylation activity at a specified nucleation region in intron 1 of *FLC* (Figure 1-2). The H3K27me3 nucleation peak is maintained during the cold and spreads along the whole *FLC* locus on return to warm, maintaining *FLC* silencing despite loss of *VIN3* expression.

It is not fully clear why increased H3K27 methylation is targeted to the nucleation region, though VAL1, which binds to the B3 domains in this region, may recruit VIN3/VRN5 and PRC2 via the ASAP complex (Questa et al., 2016). H3K36 methylation at the nucleation region is lost concomitantly with increasing H3K27 methylation, however, the two processes can be uncoupled, when there is knockdown of antisense transcripts. (Csorba et al., 2014; Mylne et al., 2006).

It is not clear whether *FLC* transcriptional shutdown permits nucleation of H3K27me3, or whether nucleation causes *FLC* transcriptional shutdown, or both. A recent model showed transcription directly antagonising PRC2 by histone exchange and association with demethylases, supporting the former (Berry, Dean, & Howard, 2017). Additionally, there is evidence that *FLC* transcriptional shutdown occurs before nucleation, independently of VIN3, indicating extensive feedback mechanisms (Swiezewski et al., 2009).

1.9 Identifying thermosensors involved in vernalisation

Despite an understanding of the events that coincide with and cause the silencing of FLC in response to vernalisation, relatively little is known about the thermosensors involved in relaying a prolonged cold temperature signal into the two processes. What is known is that, in response to prolonged cold, not only is *FLC* downregulated but components from both processes are transcriptionally induced. Total *COOLAIR* is induced in early prolonged cold peaking at three weeks whilst *VIN3* is induced only after three weeks of cold (Zhu, Rosa, & Dean, 2015) (Figure 1-5).

Increased expression of *COOLAIR* and *VIN3* is not sufficient for *FLC* silencing, given that in a line expressing high levels of both *COOLAIR* and *VIN3*, *FLC* is not epigenetically silenced (Zhao and Dean, unpub). This increased expression can however be used as a phenotype to identify thermosensors involved in the induction of *COOLAIR* and *VIN3* to fully understand how prolonged cold leads to silencing of *FLC*. So far thermosensors affecting *FLC* silencing have not been identified, although they have been modelled.

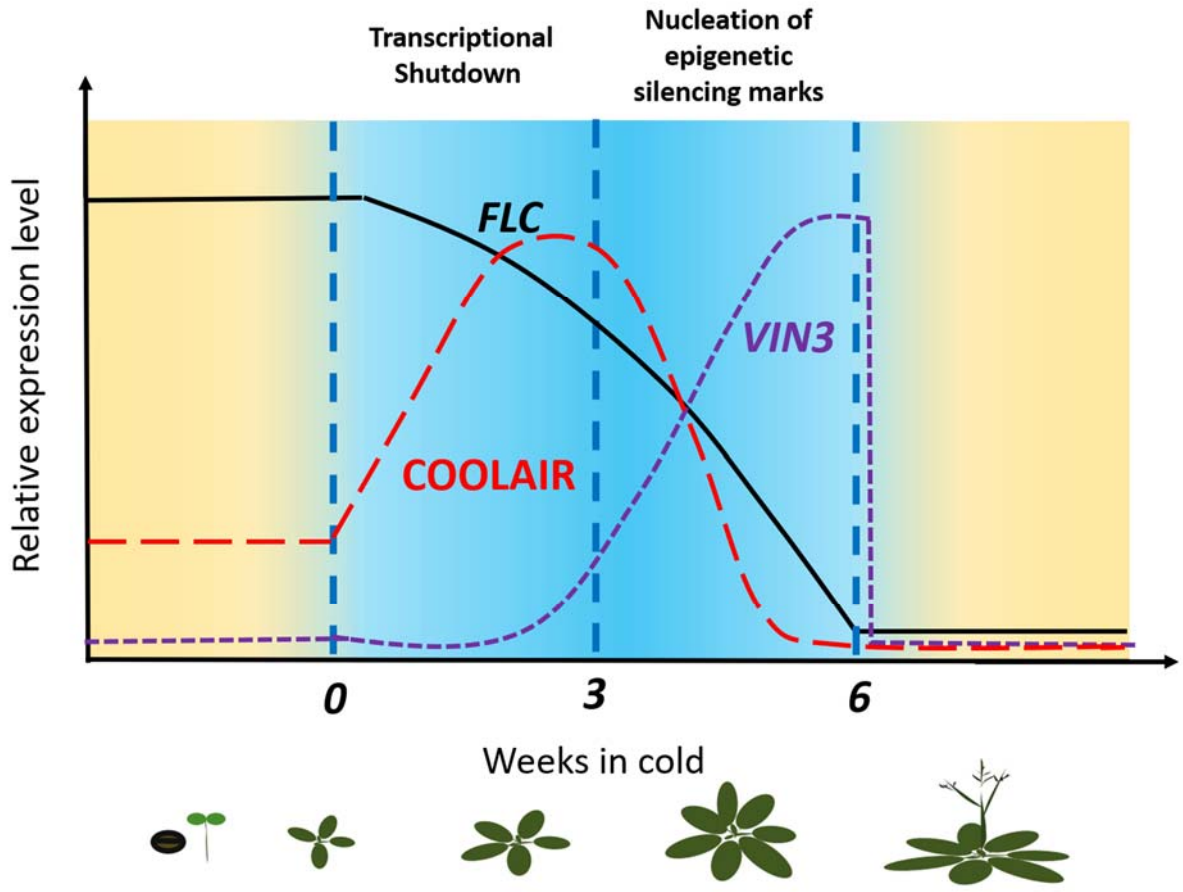


Figure 1-5 - *FLC* is transcriptionally downregulated whilst *COOLAIR* and *VIN3* are transcriptionally upregulated by prolonged cold As *FLC* levels drop during prolonged cold *COOLAIR* levels are induced by early weeks of cold and *FLC* is transcriptionally shutdown and *VIN3* is induced after three weeks of cold as nucleation of silencing marks happens at the *FLC* locus.

1.10 Modelling temperature inputs to vernalisation

In plants, various environmental signals are detected by specific proteins or families of proteins. Phytochromes detect wavelengths of light and specific resistance proteins detect the presence of specific pathogens (Richard, Gratias, Meyers, & Geffroy, 2018; Rockwell & Lagarias, 2010). Temperature has similarly been thought to be detected by a core group of thermosensors (Knight & Knight, 2012; Quint et al., 2016; Wigge, 2013). Recently, however, modelling of temperature inputs

during vernalisation has revealed a distributive thermosensor model (Antoniou-Kourounioti et al., 2018).

This model was created using vast experimental datasets of *FLC* and *VIN3* expression, in lab and also field conditions in Norwich and North and South Sweden. This mathematical model was then used to successfully predict *FLC* and *VIN3* expression levels in further field experiments in Norwich and North and South Sweden when provided with only temperature data for that year. The inputs to this model can therefore replicate *FLC* and *VIN3* expression data implying that the thermosensors suggested by the model likely exist in nature.

In this model, multiple thermosensors are required to replicate the vast experimental data available for *VIN3* and *FLC* expression patterns in response to different temperature regimes. *VIN3* slowly accumulates during prolonged cold but rapidly decreases in response to warm temperatures therefore a thermosensor measuring cold exposure over months (Long-term) and a thermosensor measuring warm temperature over hours (Current) must exist (Figure 1-6) (Antoniou-Kourounioti et al., 2018; Sung & Amasino, 2004). Additionally, *VIN3* levels decrease and remain low for the remainder of the day in response to a spike of warm temperature (Hepworth et al., 2018). *VIN3* levels recover the day after if the spike is not experienced again therefore a thermosensor measuring the occurrence of warm temperature across a day is required (Short-term)(Antoniou-Kourounioti et al., 2018). Given constitutive expression of *VIN3* cannot cause epigenetic silencing of *FLC*, cold must also affect how VIN3 and the PRC2 complex nucleate silencing marks at *FLC* (VIN3-dependent).

Overall then at least four thermosensors input into the nucleation of epigenetic silencing marks at *FLC*. Three of these are required to control the dynamics of *VIN3* expression whilst one is required to affect the nucleation process itself. The suggested thermosensors are theoretical as no thermosensors affecting VIN3 expression have been identified in Arabidopsis.

In addition to the four thermosensors required to control temperature-dependent *VIN3* expression and action, the VIN3-independent process, which is interchangeable with the transcriptional silencing of *FLC*, requires another thermosensor, as evidenced by *FLC* expression dynamics in *vin3* mutants.

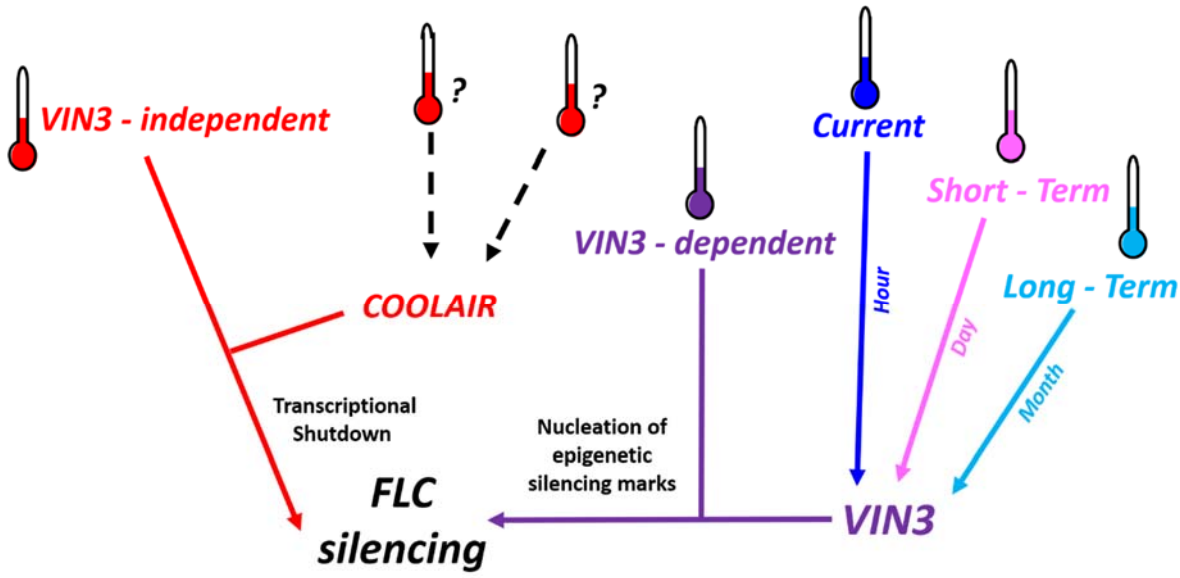


Figure 1-6 – Multiple thermosensors are required to model *FLC* and *VIN3* transcript behaviour The nucleation of epigenetic silencing marks at *FLC* theoretically requires four thermosensors. Thermosensors, measuring the 'Current', 'Short-term' and 'Long-Term' temperature, are required to reproduce VIN3 expression data under different temperature regimes whilst a further thermosensor is required to control how VIN3 causes nucleation (VIN3 – dependent). The VIN3 – independent pathway is regulated by at least one thermosensor. *COOLAIR* is part of the VIN3-independent pathway but is not regulated by the same thermosensor. It is likely upregulated in response to cold temperature by multiple unknown thermosensors. Adapted from (Antoniou-Kourounioti et al., 2018)

Based on *FLC* expression in constant, fluctuating and spike temperature experiments, the VIN3-independent pathway is likely to be activated by cold temperatures specifically at night (Antoniou-Kourounioti et al., 2018). Total *COOLAIR* expression did not correlate with this prediction but the VIN3-independent thermosensor modelled here could act through VAL1 or breakage of the gene loop, other events that are also involved in FLC transcriptional shutdown.

1.11 *COOLAIR* induction is likely controlled by multiple, unknown thermosensors

COOLAIR was originally observed to be induced in response to two weeks of lab vernalisation conditions (constant 5°C, 8-hr light/ 16-hr dark). *COOLAIR* induction is concomitant with downregulation of *FLC* sense transcripts as identified using a high-resolution array spanning the locus (Swiezewski et al., 2009). *COOLAIR* is also induced in the field in response to winter conditions, remaining high over a longer period of time, decreasing only when VIN3 levels accumulate and presumably the whole *FLC* locus is silenced (Hepworth et al., 2018).

Surprisingly, from expression data of *Arabidopsis* grown in the field across a Swedish winter, total *COOLAIR* is upregulated more over hours in response to sub-zero conditions relative to 5˚C temperatures. This result has been repeated in cabinets programmed to replicate the week in Sweden (Hepworth, Antoniou-Kourounioti, Doughty, Heckmann, Berggren, Selga, Tudor, Bloomer, Cox, Collier Harris, Yates, Wu, Irwin, Säll, Holm, Howard and Dean, in prep), in prep).

VIN3 levels are affected on different timescales by different temperature regimes similarly to *COOLAIR* levels that respond to hours of freezing temperatures and days and weeks of cold temperatures. This likely highlights the role of multiple thermosensors in the control of *COOLAIR* expression (Figure 1-4).

1.12 My Project

1) **Identification of regulators of cold-induced *COOLAIR* expression** There are currently no known candidate regulators for cold-induced *COOLAIR* expression, or even in fact general cold temperature thermosensors in *Arabidopsis thaliana*. Chapter 3 describes my attempts to identify some candidates by both a forward and reverse genetic approach.

2) **Dissection of *COOLAIR* induction in the identified mutants.** Chapter 4 describes how the identified factors affect induction of *COOLAIR* in response to prolonged cold.

3) **Investigation of *COOLAIR* induction in response to different temperature regimes** *COOLAIR* is known to increase in response to two weeks of prolonged cold and in response to short – term freezing conditions. Chapter 5 describes my investigations of *COOLAIR* induction in response to freezing conditions and fluctuating 5˚C conditions. This chapter contains information and data that may form part of a publication (Hepworth, Antoniou-Kourounioti, Doughty, Heckmann, Berggren, Selga, Tudor, Bloomer, Cox, Collier Harris, Yates, Wu, Irwin, Säll, Holm, Howard and Dean, in prep).

2 Materials and Methods

2.1 Plant Materials

All *Arabidopsis thaliana* lines are in the Col-0 or the Col FRI (sf2) background (Lee & Amasino, 1995). Col-0 plants do not require vernalisation to accelerate flowering whereas Col FRI lines carry an active FRIGIDA allele, which upregulates *FLC* in the warm to levels that repress flowering without vernalisation.

A single copy, homozygous *COOLAIR*: LUC control line was selected after transformation of Col FRI. This line is referred to as CTL throughout. Seeds from this CTL line were EMS mutagenized and over 6000 mutants were screened by imaging luciferase activity. This work was carried out by Hongchun Yang and Congyao Xu of the Dean lab.

cbf1,3 and *cbfs* mutants are loss of function CRISPR-Cas9 generated lines in the Col-0 background kindly gifted from Shuhua Yang (Jia et al., 2016).

arp6 – 1 mutants are T-DNA loss of function *arp6* lines in the Col-0 background (Deal, Kandasamy, McKinney, & Meagher, 2005).

2.2 Plant Growth conditions

For experiments presented in Chapter 3 and 4

Seeds were stratified at 5°C for three days and grown on MS media plates without glucose for seven days at 22°C in a 16-hr photoperiod (light intensity ~85umol/m^2/s). Non-vernalised plants remained in long days for an additional three days whilst vernalised plants were moved to 5°C with an 8-hr photoperiod (light intensity ~18umol/m^2/s) specified lengths of time.

For the freezing experiment in Chapter 5

Seeds were sown on netting over soil, to reduce soil transfer to samples, and stratified at 5°C for three days. Non-vernalised plants were grown for 10 days in a 16-hr light 22°C / 8-hr dark 20°C growth chamber (light intensity ~220umol/ m^2/s). Plants were then placed into 8-hr light 5°C (light intensity ~18umol/m^2/s) / 16-hr dark 5°C conditions for four days to acclimatise to cold

temperatures. Plants then remained in these conditions for one further day or were transferred to 8-hr light -0.5°C (light intensity ~18mol/m^2/s) /16-hr dark -0.5°C freezing conditions.

For the fluctuating temperature experiments in Chapter 5

Seeds were sown onto soil and stratified at 5°C for three days. Non-vernalised plants were grown for 10 days in a 16-hr light 22°C / 8-hr dark 20°C growth chamber (light intensity 220umol//m^2/s). Plants were then moved to 8-hr photoperiod (light intensity ~18umol/m^2/s) chambers with three different temperature regimes: constant 5°C, Norwich fluctuating 5°C (Range 3°C to 7°C) and Sweden fluctuating 5°C (Range -1°C to 12°C) as depicted in the Results section.

2.3 Luciferase Imaging

Arabidopsis were grown as above, and at the relevant time point, 1mM luciferin (potassium salt, Promega, Chilworth, UK) solution was sprayed onto the seedlings. Plates were placed in the dark for 15 minutes before luciferase activity was monitored over a one-minute exposure using a Night Owl light-sensitive CCD camera (Berthold Technologies, Harpenden, UK) to capture the bioluminescence signal.

2.4 Sequence Alignment

Genome sequences were obtained from Ensemble plants. Sequences 1kb downstream from the *FLC* STOP codon site in each species were collected and multiple aligned using Geneious R7 (https://www.geneious.com/).

2.5 qPCR expression analysis

cDNA was synthesised from 1500 – 2500ng of extracted total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen, CA, US) using oligo dT (Sigma, Haverhill, UK) for *CBF* expression analysis or gene specific primers previously used by the lab (Hepworth et al., 2018), for *FLC*, total *COOLAIR,* proximal *COOLAIR* and *LUCIFERASE* expression analysis. In experiments where *FLC* and *COOLAIR* expression were both measured two separate RT reactions were set up, one with *FLC* RT primers and the other with *COOLAIR* RT primers to prevent amplification of sense and antisense transcripts at the same time. cDNA was amplified using a LightCycler® 480 (Roche Technologies, Basel, Switzerland)

and SYBR Green I Master mix (Roche) to detect product accumulation. Three technical replicates were carried out for each sample**.**

Relative expression values were calculated by averaging the three technical replicates and normalising each of these values to the geometric mean of the two control genes, UBC and PP2A, whose expression does not change with cold exposure (Hepworth et al., 2018). This was achieved using the Pfaffl method (Pfaffl, 2001) using primer efficiencies determined by LinRegPCR, a program that uses linear regression to determine the efficiency of primers in each well and calculates an overall primer efficiency by averaging the calculated efficiencies across all wells containing this primer set (Ruijter et al., 2009).
(https://www.gene-quantification.de/LinRegPCR_help_manual_v11.0.pdf , http://download.gene-quantification.info/)

2.6 Data analysis

In all experiments, values presented are the mean of 2-3 biological replicates. These biological replicates were grown at the same time on different plates and harvested for expression analysis at the same time. Error bars shown are the standard error of the mean. Where applicable, student's t – test and ANOVA have been used to determine the statistical significance of any findings. Tukey's post hoc test has been used to determine which groups differ when an ANOVA test reveals significant differences.

3 Identification of regulators of cold-induced *COOLAIR* expression

3.1 Background

COOLAIR, the set of lncRNAs antisense to *FLC,* are induced by weeks of cold: constant 5°C if grown in vernalisation lab conditions (Swiezewski et al., 2009). *COOLAIR* induction is coincident with downregulation of spliced and unspliced *FLC* and is likely required for this transcriptional shutdown of *FLC* (Csorba et al., 2014; Swiezewski et al., 2009). It is unknown how cold temperature induces *COOLAIR* and so, to identify the pathway and importantly the thermosensor responsible, a forward genetic mutant screen was undertaken.

As *COOLAIR* transcripts are not translated in wild-type plants, a modified gene fusion was created whereby the *COOLAIR* promotor, as defined as the region upstream of the neighbouring gene, *COOLAIR* exon 1, intron 1 and a small segment of exon 2 was fused to the cDNA of firefly luciferase and an internal ribosome entry site (Figure 3-1A) (Sun et al., 2013). This *COOLAIR*: LUC reporter does not produce *FLC* sense transcripts meaning complex feedback between sense and antisense transcription is not present.

Col FRI plants carrying wild-type copies of *FLC*, and the associated endogenous *COOLAIR* transcripts, were transformed with the *COOLAIR*: LUC construct. Multiple lines displayed a *COOLAIR*: LUC phenotype that replicated the endogenous *COOLAIR* phenotype in response to prolonged cold. *COOLAIR*: *LUC* transcript level was increased by exposure to 18 days of vernalisation conditions (Figure 3-1B). A single copy insertion line that, in addition to inducing *COOLAIR*: LUC in response to vernalisation conditions, displayed wild-type like expression of endogenous *FLC* and *COOLAIR* expression was selected and EMS mutagenized. Mutants were screened by luciferase imaging in the M2 generation and screened again in the M3 generation before cold, (NV) and after 18 days of cold exposure (V) (Figure 3-1C).

Mutants were classified as dark (D) or bright (B) at two different time points after 10 days pre-growth (NV) or after 7 days pre-growth and 18 days in vernalisation conditions (V). Mutants were classified as V mutants only if there was no NV phenotype. Mutants can therefore be classified into four groups; Non-vernalisation dark (NVD), non-vernalisation bright (NVB), vernalisation dark (VD) and vernalisation bright (VB) based on the altered *COOLAIR*: LUC dynamics displayed (Figure 3-1D).

For example, in Figure 3-1C, mutant A would be classified as an NVB mutant given the higher luciferase activity displayed under NV conditions relative to the CTL line. Mutant C would be classed as an NVD mutant given the highly reduced levels of luciferase activity under NV conditions. Mutant D would

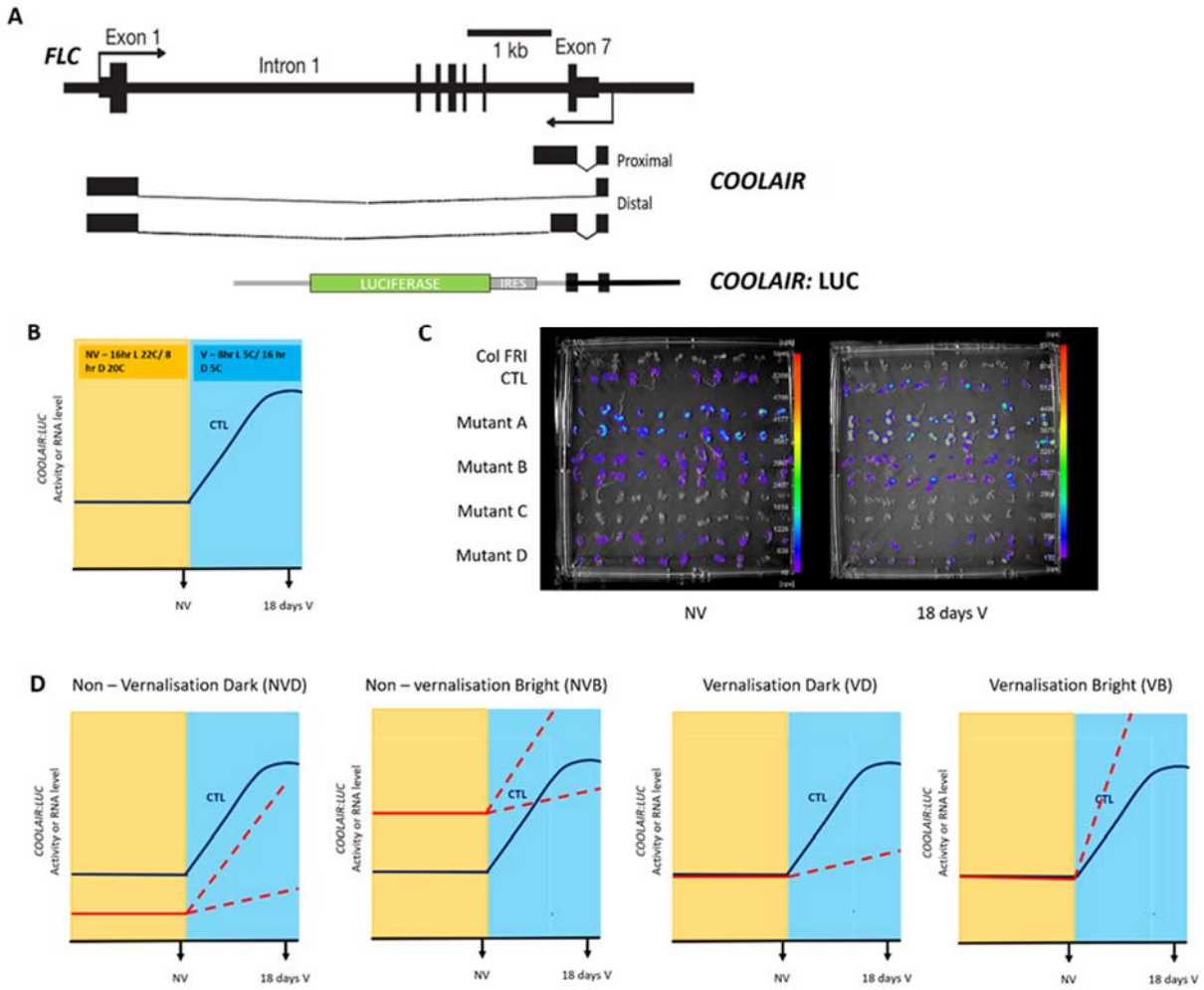


Figure 3-1 – *COOLAIR: LUC* as a readout for *COOLAIR* expression A) The *FLC* locus shows the exons of *FLC* in the sense direction and the different *COOLAIR* forms in the antisense direction. *COOLAIR: LUC* was created by fusing luciferase cDNA to the promotor and initial sequence of *COOLAIR*. B) The CTL line contains *COOLAIR: LUC* which is induced by 18 days of vernalisation mimicking endogenous *COOLAIR*. C) CTL seeds were EMS mutagenized and over 6000 mutants screened for luciferase activity under non-vernalisation (NV) and vernalisation (V) conditions. D) Mutants were categorised as Non-vernalisation dark (NVD), non-vernalisation bright (NVB), vernalisation dark (VD) or vernalisation bright (VB) based on the *COOLAIR: LUC* phenotype. Red lines represent the potential mutant phenotypes displayed in each type of mutant.

be classified as an VD mutant given the similar luciferase activity under NV conditions but the reduced luciferase activity observed in response to vernalisation. Mutant B would not be classified as having a mutant phenotype.

Given the work in this thesis aims to identify factors required for cold induction of *COOLAIR*, VD mutants were of interest as these mutants would correspond to plants that had lost function of a cold-inducible *COOLAIR* activator (Figure 3-1D, VD graph). Additionally, some NVD and NVB mutants could also be interesting to follow up as they may be impaired in *COOLAIR*: LUC induction in addition to possessing altered NV *COOLAIR*: LUC starting levels (Figure 3-1D). For example, NVB mutants could represent plants that had lost function of a cold-repressed *COOLAIR* repressor, a factor that usually represses *COOLAIR* induction except under vernalisation conditions.

Forward genetic mutant screens are a genome-wide, unbiased way to identify candidate factors important for a specific phenotype, in this case the cold induction of *COOLAIR*. Forward mutant screens however have various limitations including the low likelihood of identifying factors that are redundantly involved in the same process. Given the evidence that thermosensors may be distributive, *COOLAIR* induction could likely be controlled by multiple pathways and factors (Antoniou-Kourounioti et al., 2018). To combat this, an alternative approach investigating cis elements present in the *COOLAIR* promotor was used to identify potential trans-binding *COOLAIR* regulators that may have redundancy. This parallel approach identified known redundant cold-affected factors.

Whilst the forward genetic mutant screen was originally set up and performed by Hongchun Yang and later Congyao Xu, I was responsible for confirming luciferase and gene expression phenotypes in a subset of the M3 generation, including mutants 6755 and 6722. Luciferase and gene expression phenotypes and characterisation of mutants 2265 and 2273 was completed by Hongchun Yang. I independently searched for cis elements in the *COOLAIR* promotor.

3.2 Aim

To Identify regulators of cold-induced *COOLAIR* expression

3.3 Results

3.3.1 No VD mutants were identified from the screen

Intuitively, one might expect that screening for cold-specific inducers of *COOLAIR: LUC* expression would reveal mutations in cold-induced activators of *COOLAIR*. These vernalisation dark (VD)

mutants would have CTL levels of luciferase activity in the warm but would be impaired in the increase in luciferase activity expected in the cold (Figure 3-1D). Surprisingly, of over 6000 individual mutant lines screened, no VD mutants were found. Whilst some lines may have had a VD luciferase activity phenotype, qPCR analysis of *COOLAIR*: LUC levels identified them as NVD mutants (data not shown).

3.3.2 NVD mutants were not investigated closely

As mentioned some NVD mutants may also be impaired in *COOLAIR*: LUC induction in addition to having low starting *COOLAIR*: LUC levels. Multiple NVD mutants were identified as having mutations in the luciferase reporter whilst many mutants that showed no luciferase expression at all were shown to be lacking the reporter gene altogether, likely a result of seed contamination. Given the high prevalence of the NVD mutants they were not investigated further in order to direct time and resources to NVB mutants.

3.3.3 Some NVB mutants show an altered induction of *COOLAIR: LUC*

The majority of mutants identified from the screen form the NVB group. These mutants showed higher levels of *COOLAIR:* LUC before cold (Figure 3-1D). Many of these mutants still showed induction of *COOLAIR: LUC* in response to cold and were not interesting to follow up given that this thesis aims to identify cold-affected *COOLAIR* regulators. Some of the NVB mutants did however possess an impaired induction of *COOLAIR* in response to cold so were good candidate cold-affected *COOLAIR* regulators.

Figure 3-2 shows the induction of *COOLAIR: LUC* in various NVB mutants. All expression values have been normalised to the NV level of each genotype meaning that the blue vernalisation treatment bars represent the fold-change in *COOLAIR* expression observed in response to cold temperature.

Mutant 6755 is a representative NVB mutant that was not affected in *COOLAIR: LUC* induction. *COOLAIR*: LUC was induced just over two-fold by vernalisation treatment in the CTL line and 6755. Mutants 6722, 2265 and 2273 showed a loss of *COOLAIR:* LUC induction in response to vernalisation. In 6722 *COOLAIR:* LUC was induced by 1.2 times compared to over a two-fold change in the CTL line. In 2265 and 2273, *COOLAIR:* LUC was induced by less than 1.2 times compared to over a 2.8-fold change in the CTL line.

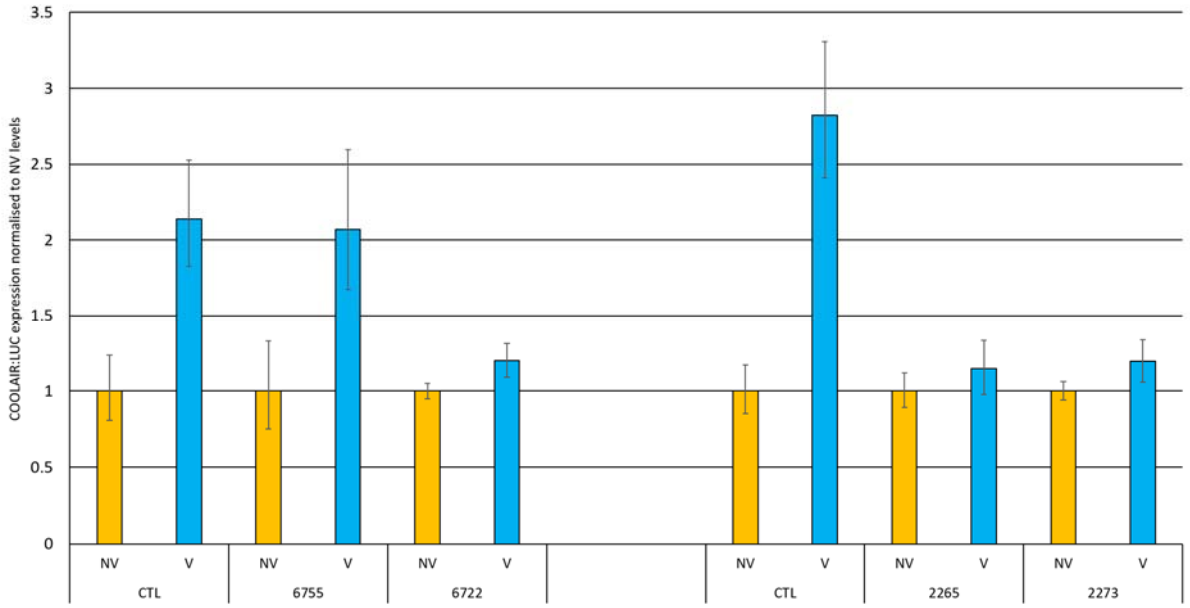


Figure 3-2 – *COOLAIR*: LUC induction is affected in some NVB mutants Expression values, as measured by qPCR, were normalised to the NV level of *COOLAIR*: LUC hence the *COOLAIR*: LUC NV levels are all represented as 1 (orange bars) in CTL and all mutants. The blue bar represents the *COOLAIR*: LUC V levels normalised to NV. All mutants shown are NVB. This graph includes two independent experiments, so CTL levels vary slightly across the graph. Error bars represent s.e.m. calculated by using the s.e.m. of the NV and V expression levels. Student's t- test not performed due to the normalisation calculation.

3.3.4 NVB mutants with impaired *COOLAIR*: LUC induction, 6722 and 2265, had different *FLC* and proximal *COOLAIR* phenotypes

6722 and 2265 were both NVB mutants (Figure 3-3A, 4A) with impaired *COOLAIR*: LUC induction (Figure 3-2). Compared to CTL plants, the NV level of *COOLAIR*: LUC RNA in both 6722 and 2265 was four-fold higher and significantly different (Student's t-test, n=3, $p<0.05$) (Figure 3-3B, 3-4B). This is even higher than the level of *COOLAIR*: LUC after exposure to cold temperatures in the CTL line. In 6722 and 2265, *COOLAIR*: LUC was only slightly induced by exposure to vernalisation.

Given the similarity of the behaviour of 6722 and 2265 it was interesting to analyse other expression phenotypes of these two NVB mutants. In 6722, spliced *FLC* levels were remarkably similar to the Col FRI and CTL line at NV and V time points and not significantly different (Student's t-test, n=3, $p>0.05$ at NV and V) (Figure 3-3C). In contrast, spliced *FLC* was reduced three-fold at NV in 2265 compared to the CTL line, a significant difference (Student's t-test, n=3, $p<0.05$) (Figure 3-4C).

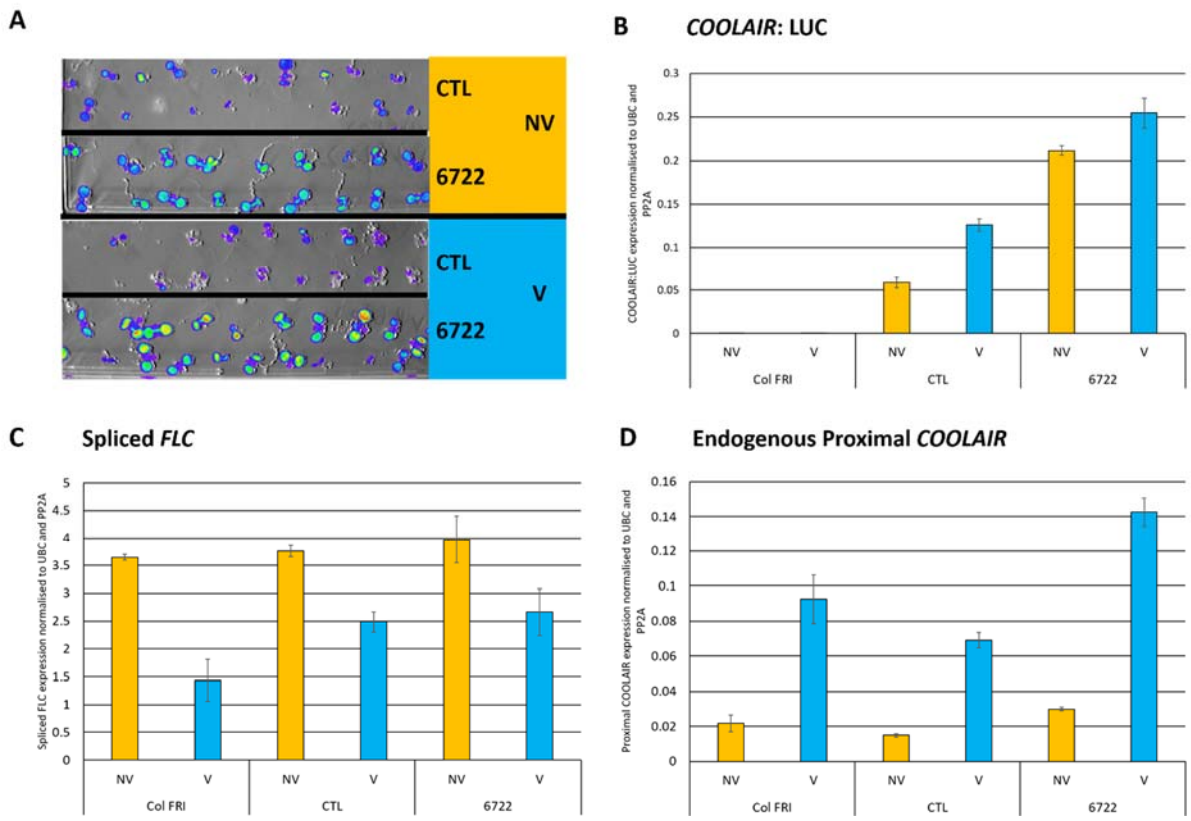


Figure 3-3 – NVB mutant 6722 phenotypes A) Luciferase activity of *COOLAIR*: LUC compared to CTL under NV and V conditions**.** Expression level under NV and V conditions of B) *COOLAIR*: LUC, C) Spliced *FLC* and D) Endogenous proximal *COOLAIR*. Bars represent means of three biological replicates. Error bars represent s.e.m. Student's t-test comparing CTL and 6722 expression phenotypes at NV and V time points separately reveal significant differences in Luc expression NV ($p<0.001$) and V ($p<0.005$) and proximal COOLAIR expression NV ($p<0.001$) and V ($p<0.01$) but no significant differences in spliced FLC expression NV and V ($p>0.05$).

Endogenous proximal *COOLAIR* was two-fold higher NV in 6722 compared to the CTL line, a significant difference (Student's t-test, n=3, $p<0.001$). This trend supported the effect of the 6722 mutation on *COOLAIR*: LUC levels NV. After vernalisation, proximal *COOLAIR* remained two-fold higher in 6722 compared to the CTL line, again significantly different (Student's t – test, n=3, $p<0.01$) (Figure 3-3D). Therefore, despite higher starting levels of endogenous proximal *COOLAIR*, induction of proximal *COOLAIR* in response to cold was unimpaired in 6722. This was different to the *COOLAIR*: LUC phenotype whereby *COOLAIR*: LUC was induced less by cold in 6722.

In 2265, endogenous proximal *COOLAIR* was reduced to just above 60% of the CTL line level at NV (Figure 3-4D), a significant difference (Student's t-test, n=3, $p<0.05$). This was opposite to the *COOLAIR*: LUC phenotype observed at NV. In 2265, *COOLAIR*: LUC was higher NV whereas endogenous proximal *COOLAIR* was reduced relative to the CTL line. Induction of proximal *COOLAIR*

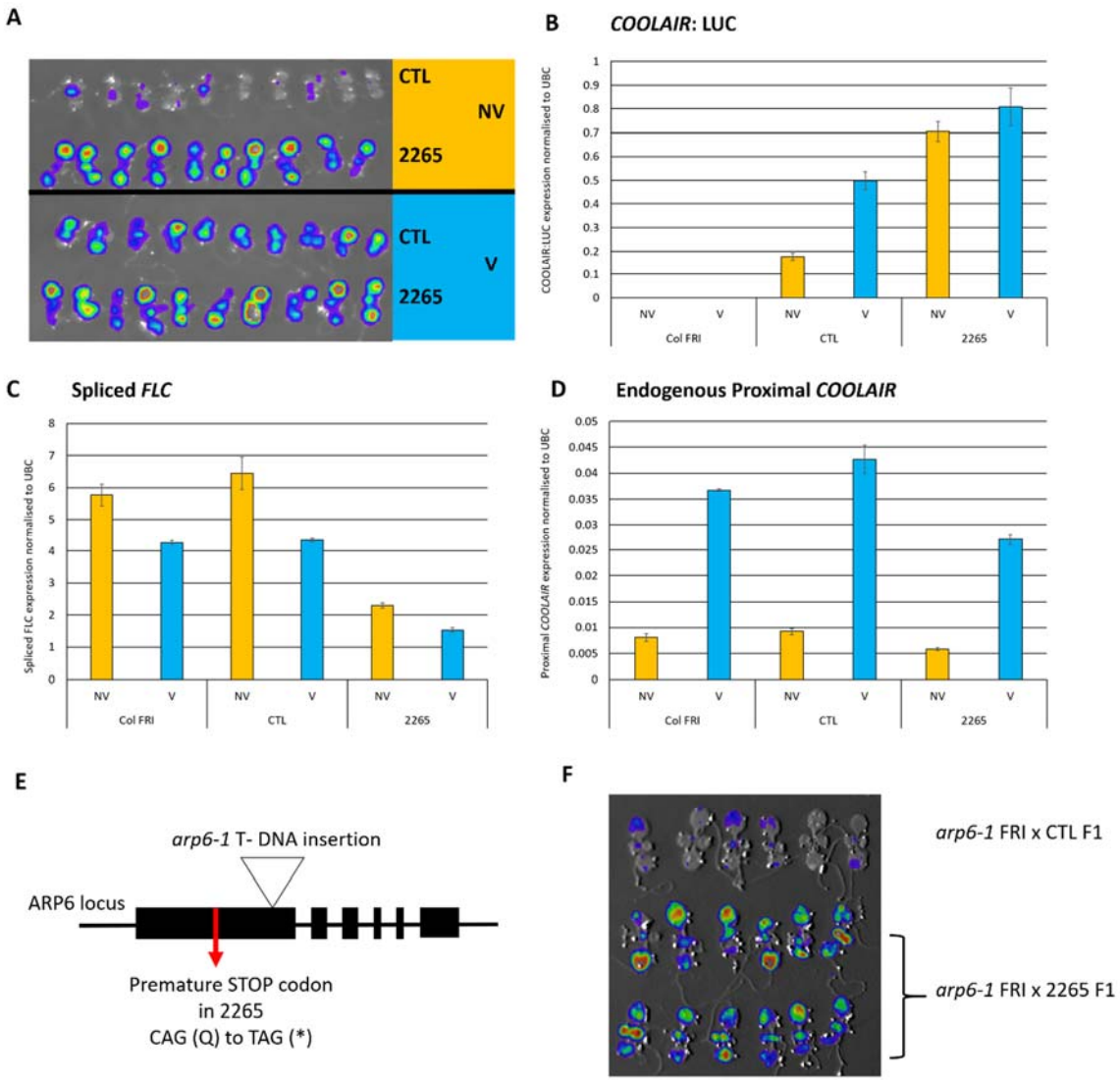


Figure 3-4 – NVB mutant 2265 identified as *arp6* A) Luciferase activity of *COOLAIR*: LUC compared to CTL under NV and V conditions**.** Expression level under NV and V conditions of B) *COOLAIR*: LUC, C) Spliced *FLC* and D) Endogenous proximal *COOLAIR*. Bars represent means of three biological replicates. Error bars represent s.e.m. Student's t-test comparing CTL and 2265 expression phenotypes at NV and V time points separately reveal significant differences in Luc expression NV ($p<0.001$) and V ($p<0.05$) and proximal COOLAIR expression NV ($p<0.05$) and V ($p<0.05$) and spliced FLC expression NV ($p<0.05$) and V ($p>0.0001$). E) Diagram depicting the causative mutation in ARP6 in NVB mutant 2265. F) Complementation crosses reveal *arp6-1* cannot rescue the 2265 mutant phenotype relative to luciferase activity. All experimental work presented in this figure was carried out by Hongchun Yang.

in response to cold was unimpaired in 2265, induced 4.5 times in 2265 and the CTL line. The resultant endogenous proximal *COOLAIR* level at V was therefore just above 60% of the CTL line.

Overall, 2265 and 6722 had very similar *COOLAIR*: LUC phenotypes, NV and V, when compared to the CTL line however they had very different *FLC* and endogenous proximal *COOLAIR* expression

phenotypes. Likely then 2265 and 6722 represent mutations in different targets providing two candidate cold-affected *COOLAIR* regulators.

3.3.5 An NVB mutant with impaired endogenous proximal *COOLAIR* induction, 2265, is a loss of function *arp6* allele

2265 was crossed back to the CTL line and the resulting F3 lines were screened for the NVB luciferase phenotype and the DNA sent for sequencing to map the EMS mutation responsible for the phenotype.

A post doc in the lab, Hongchun Yang, identified NVB mutant 2265 as a loss of function *arp6* mutant. A premature STOP codon had been introduced by a C to T mutation in exon 1 of ARP6 (Figure 3-4E).

Complementation crosses revealed that the observed NVB luciferase phenotype is very likely due to this mutation (Figure 3-4F). Backcrossing 2265 with *arp6-1*, a T-DNA mutant, could not rescue to the luciferase phenotype compared to crossing *arp6-1* with the CTL line.

3.3.6 Redundant factors bind conserved elements of the *COOLAIR* promotor *in vitro*

Alongside the forward genetic mutant screen, a DNA Affinity Purification (DAP) - seq database was investigated to identify potential factors that bind the *COOLAIR* promotor *in vitro* (O'Malley et al., 2016) (http://neomorph.salk.edu/aj2/pages/hchen/dap_ath_pub_models.php). DAP-seq identifies binding of immobilised transcription factors to genomic DNA (Bartlett et al., 2017). In this data set, 529 *Arabidopsis* transcription factors were tested against the *Arabidopsis* genome. NTL8, CBF1 and CBF3 but not CBF2, and CAMTA1 but not CAMTA5 all bind the *FLC* 3' region, the equivalent of the *COOLAIR* promotor, *in vitro* (Figure 3-5A).

The motifs of these transcription factors were identified within the *COOLAIR* promotor. Two CRT/DRE elements, with a CCGAC/GTCGG core sequence known to bind all three CBF factors, was found most downstream of the *FLC* stop codon. This core motif was fully conserved across five Brassicaceae species based on alignment of the 1kb sequence downstream of the *FLC* stop codon. A CAMTA1 binding site, with the core motif CGCGT, was also conserved across four of the five Brassicaceae species, diverging between *Arabidopsis thaliana* and *Arabidopsis lyrata*. The NTL8

binding site (NTTNCTTCNNNNNNAAGNA/TNCTTNNNNNNGAAGNAAN) was also conserved across all five Brassicaceae species. Divergence was observed within the motifs at N positions highlighting perhaps that mutation is occurring in this region but important bases within the motif have been conserved.

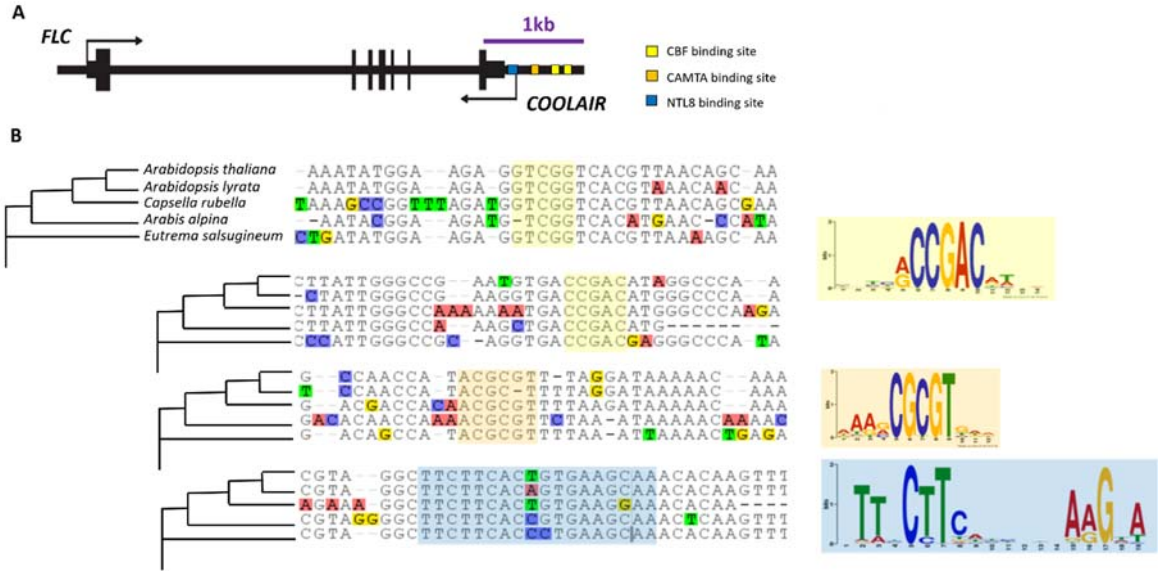


Figure 3-5 – Trans factor binding sites in the *COOLAIR* promotor A) *FLC/ COOLAIR* locus labelled with binding sites of Arabidopsis thaliana transcription factors identified by DAP-seq (Bartlett et al., 2017; O'Malley et al., 2016). The 1kb base sequence downstream of the *FLC* stop codon was aligned from five Brassicaceae species B) revealing conservation of these binding sites. The binding motifs are shown on the right. Yellow – CBF1,2,3 binding site, Orange – CAMTA1 binding site, Blue – NTL8 binding site.

3.4 Discussion

3.4.1 Few mutants impaired in *COOLAIR*: LUC induction could be isolated

No VD mutants could be recovered from the screen highlighting that a single cold-inducible activator of *COOLAIR* expression does not exist. This could be due to several reasons.

This may be because *COOLAIR*: LUC is induced through a different mechanism. For example, a repressor of *COOLAIR*: LUC may be inactivated in response to cold temperature. This is somewhat supported by the recovery of NVB mutants with impaired *COOLAIR*: LUC induction such as 6722, 2265 (*arp6*) and 2273. In these mutants *COOLAIR*: LUC is elevated under NV conditions, above the level of *COOLAIR*: LUC induced by cold in the CTL line. *COOLAIR*: LUC is then not induced as much by exposure to vernalisation conditions.

Additionally, cold-inducible activators of *COOLAIR*: LUC may exist however there may be redundancy between a family of proteins. In this case, the likelihood of knocking out all family members is so low that the VD mutant would never be recovered. This is somewhat supported by the evidence of trans factor binding the *COOLAIR* promotor that are known to be part of known redundant families. CBF1,2 and 3 are redundant cold induced transcription factors whilst CAMTA1,2,3,4,5 and 6 have certain levels of redundancy that have not been fully elucidated. NTL8 is also part of a group of three NTL proteins, including NTL14 and NTL5 that may also show redundancy. In all these three cases, potential transcriptional activators are part of larger redundant families.

Finally, there is a chance that the changes introduced by creating a translatable reporter of a lncRNA has changed the pathways important for induction. LncRNAs and protein coding genes have alternative associated expression control mechanisms linked to the different fates of the RNA (Schlackow, Nojima et al. 2016, Mele, Mattioli et al. 2017). The inclusion of exon 1 and intron 1 and segment of exon 2 of the *COOLAIR* lncRNA aimed to combat these differences and the behaviour of luciferase mRNA is consistent with endogenous *COOLAIR* behaviour in response to cold.

3.4.2 ARP6 is implicated in *COOLAIR*: LUC induction

2265, an NVB mutant with impaired *COOLAIR*: LUC induction was identified as a loss of function mutant in ARP6. ARP6 is an integral subunit of the C-module of the Arabidopsis SWR1 complex

(Gerhold and Gasser 2014). This complex, conserved from yeast, deposits a histone variant, H2A.Z, in nucleosomes.

ARP6 has already been implicated in the control of *FLC* expression, originally identified in a screen for early flowering in short days. In the various *arp6* mutants, *FLC* transcripts are reduced and flowering gene transcripts such as FT are increased (Martin-Trillo, Lazaro et al. 2006, Deal, Topp et al. 2007). *arp6-1* is a suppressor of *fca*, an autonomous pathway component, and partial suppressor of FRI implying that ARP6 is required for the high levels of FLC expression seen in these backgrounds (Martin-Trillo, Lazaro et al. 2006). Mutants in other components of the SWR1 complex, including *pie1*, *swc6* and *sef2* have an early flowering phenotype due to downregulation of *FLC* (Choi, Park et al. 2007; Deal, Topp et al. 2007; Lazaro, Gomez-Zambrano et al. 2008; Yun, Hyun et al. 2011). Although the pleiotropic phenotypes of the different mutants are similar, there are some differences highlighting functional differences between the components.

COOLAIR expression has not been studied in the *arp6* mutants described above so it is interesting that *arp6* was isolated as an NVB *COOLAIR*: LUC mutant. Given the complex feedbacks between sense and antisense transcription at the *FLC* locus, this discovery could show that ARP6 is primarily important for impacting *COOLAIR* expression, which in turn affects *FLC* expression, or that ARP6 directly regulates both *FLC* and *COOLAIR*.

The latter may be more likely given that H2A.Z is present at the 5' and 3' ends of *FLC* and this is lost in the *arp6-1* mutant (Deal, Topp et al. 2007). At the 5' end H2A.Z maps to the +1 nucleosome whereas at the 3' end H2AZ maps to *COOLAIR* exon 2 near various splice sites of the different COOLAIR forms (Figure 3-6). In *arp6* (2265), whilst *COOLAIR*: LUC is elevated NV, both *FLC* and endogenous proximal *COOLAIR* are repressed NV, perhaps as a result of competing expression levels of *FLC* and *COOLAIR* that ultimately correlate on a whole plant level.

In *arp6* (2265), in response to vernalisation, *COOLAIR*: LUC is induced less. In other words, the *arp6* mutation is epistatic to cold temperature. In genome-wide studies, H2A.Z has been linked to antisense RNA repression, transcriptional activation, transcriptional repression and interestingly temperature sensing (March-Diaz, Garcia-Dominguez et al. 2008; Zofall, Fischer et al. 2009; Kumar and Wigge 2010; Coleman-Derr and Zilberman 2012; Sura, Kabza et al. 2017). H2A.Z has shown to be lost from chromatin at high temperatures, negatively or positively affecting gene expression dependent on whether this accessibility allows binding of transcriptional repressors or activators

(Kumar and Wigge 2010). It is unknown how H2A.Z distribution changes in response to cool temperatures such as those experienced during vernalisation. H2A.Z at *COOLAIR*: LUC may therefore be repressive except in response to cold temperature where it may be redistributed or modified in a way that permits *COOLAIR*: LUC expression. In an *arp6* mutant, loss of H2A.Z pre-induces *COOLAIR*: LUC and vernalisation has no further effects.

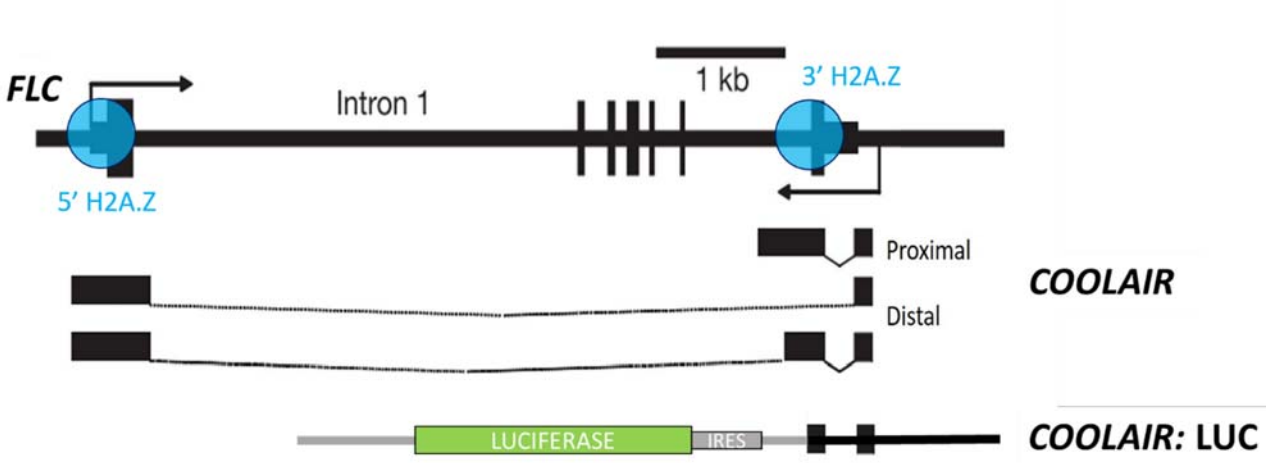


Figure 3-6 – H2A.Z distribution at the FLC locus H2A.Z is enriched at the 5' end of FLC at the +1 nucleosome and at the 3' end of FLC over the FLC stop codon and exon 7. This is downstream in the antisense direction of the *COOLAIR* promotor and *COOLAIR* exon 1.

3.4.3 Mutant 6722 is a candidate cold-associated regulator of *COOLAIR*: LUC

Another NVB mutant with impaired *COOLAIR*: LUC induction, 6722, is a good candidate repressor of *COOLAIR*: LUC that is itself inactivated by vernalisation to permit *COOLAIR*: LUC expression. It is likely not another *arp6* mutation given the lack of an *FLC* expression phenotype.

In 6722, endogenous proximal *COOLAIR* is also elevated prior to cold, like *COOLAIR*: LUC however induction in response to cold is not impaired, unlike *COOLAIR*: LUC. This could be due to several reasons.

COOLAIR: LUC may not be further induced as the 6722 mutation causes saturation of expression at NV. At the endogenous locus this may not happen given the FLC context of *COOLAIR*. *FLC* and *COOLAIR* antagonise the expression of each other and endogenous proximal COOLAIR expression may be restrained by *FLC* expression despite the loss of a *COOLAIR* repressor.

Alternatively, other distal *COOLAIR* forms or total *COOLAIR* may be impaired in induction at the endogenous locus. Proximal *COOLAIR* may not be affected in the same way as *COOLAIR*: LUC or the other endogenous *COOLAIR* forms.

3.4.4 Known cold-affected, redundant transcription factors bind the *COOLAIR* promotor *in vitro*

Cold – inducible activators of *COOLAIR* expression may not have been isolated from the *COOLAIR*: LUC forward genetic screen due to redundancy between these factors. This idea is supported by the binding of three families of known redundant factors to the *COOLAIR* promotor in vitro, importantly binding to conserved sites. Of interest is the binding of the CBFs and CAMTAs to this promotor as both families are known to be influenced transcriptionally or post translationally by low temperatures.

C – repeat binding factors (CBF1,2 and 3) also known as dehydration response element binding factors (DREB1B, 1C and 1A) are redundant transcriptional activators that recognise CRT/DRE elements with a core CCGAC motif. CBF1,2 and 3 are well characterised master regulators of the cold acclimation transcriptome (Gilmour et al., 2004; Gilmour et al., 1998; Jaglo-Ottosen, Gilmour, Zarka, Schabenberger, & Thomashow, 1998; Stockinger et al., 1997); Knight and Knight 2012; Kim, Park et al. 2013; Kim, Lee et al. 2015; Jia, Ding et al. 2016; Zhao and Zhu 2016; Kidokoro, Yoneda et al. 2017). CBF1, 2 and 3 are transcriptionally induced by exposure to temperatures below 12°C and they activate expression of a suite of cold-responsive (COR) genes required for increased freezing tolerance after cold acclimation. Given the known induction of CBF factors in response to cold temperature and the downstream activation of many gene targets, it is interesting to investigate whether the CBF factors they may also redundantly activate *COOLAIR* expression in response to prolonged cold.

Historically, CBF expression or the expression of CBF-regulated genes has been studied in response to short-term cold temperatures usually between zero to twenty-four hours (Vogel, Zarka et al. 2005; Jia, Ding et al. 2016; Zhao, Zhang et al. 2016; Shi, Huang et al. 2017). The CBFs have been shown to be induced after as little as 15 minutes at 4°C (Shi, Ding, & Yang, 2018). It has been claimed that CBF expression is negatively regulated during prolonged cold exposure to combat the growth retardation associated with CBF expression however there is evidence that even after five weeks of cool temperature, CBF expression is higher than under warm conditions (Lee, Fleming et al. 2009; Liu, Jia et al. 2017). CBF2 expression was induced between 5 to 25-fold in italian Arabidopsis

ecotypes transformed with Italian (5-fold) or swedish (25-fold) ecotype CBF2 constructs (Gehan et al., 2015). Other transcriptomic studies reveal that the CBFs are transcriptionally induced between 3 to 6hr after exposure to cold temperature and on day 4 of cold exposure are expressed close to the level observed prior to cold exposure (Calixto et al., 2018). *COOLAIR* is induced maximally by three weeks of cold but close inspection of qPCR data reveals that *COOLAIR* is upregulated after four hours of cold and is upregulated to half the final level by 12 hours (Bloomer, unpub).

CBF expression is regulated by many transcription factors; positively by CAMTA3, ICE1, BZR1 and negatively by some PIFs, EIN3 and MYB15 and is circadian clock gated by LHY and CCA1 (Chinnusamy, Ohta et al. 2003; Dong, Agarwal et al. 2006; Jiang, Shi et al. 2017; Shi, Ding et al. 2018). These transcription factors themselves are closely regulated by the circadian clock, hormones including ethylene and brassinosteroids, light, calcium, protein kinases and developmental pathways such as flowering (Shi, Ding et al. 2018).

The cold thermosensor involved in the transcriptional induction of the CBFs has so far not been elucidated. An increase in Ca2+ occurs very early in the cold response and this Ca2+ could act as an internal secondary messenger for cold temperature (Heather Knight & Knight, 2000). It is not clear how Ca2+ is induced by cold though membrane fluidity, Ca2+ channels and the cytoskeleton have been suggested to play a role (Knight & Knight, 2012).

Interestingly, the CBFs have been associated with the flowering pathway before. Lines overexpressing CBF express *FLC* to higher levels and flowering is delayed in these plants (Seo, Lee et al. 2009). *COOLAIR* expression was never tested in these lines and it was not revealed how the CBF factors cause an increase in *FLC* expression. An attractive hypothesis could be the influence of the CBF factors on *COOLAIR* expression, which in turn feeds back onto *FLC* expression.

Another flowering-associated regulator, FVE that forms part of the autonomous pathway, has also been implicated in the cold acclimation pathway. In a *fve* mutant, COR genes are elevated without exposure of the plants to cold and without induction of CBF1,2 and 3. FVE is required at the *FLC* locus to repress *FLC* expression via the autonomous pathway (Kim, Hyun et al. 2004). Like at other CBF-targeted COR genes, FVE may repress *COOLAIR* (and *FLC*) expression until this repression is lifted by expression of CBF1,2 and 3.

Calmodulin – binding transcriptional activators (CAMTAs) encompass a family of six proteins that have conserved protein domains including a CG-1 sequence specific DNA binding domain, a TIG non – sequence specific DNA binding domain, three ankyrin repeats and five calmodulin binding domains (Pandey, Ranjan et al. 2013). The CAMTAs can be divided into three sub – types; CAMTA1,2,3, CAMTA4 and CAMTA5,6 (Kidokoro, Yoneda et al. 2017). CAMTA1, but not CAMTA5, has been implicated in binding the *COOLAIR* promotor in vitro.

In response to weeks of cold SA biosynthesis genes are transcriptionally induced to prepare for infection as a result of increased damage due to cold temperature. Interestingly this induction is not observed in a *camta1,2,3* triple mutant (Kim, Park et al. 2013). It is therefore claimed that CAMTA 1,2 and 3 redundantly repress SA biosynthesis genes and that this repression is lifted by exposure to weeks of cold temperature (Kim, An et al. 2017). This pattern of expression is analogous to induction of *COOLAIR* in response to weeks of cold and coupled with the presence of conserved CAMTA1 binding sites could implicate CAMTA1,2,3 in cold – specific induction of *COOLAIR*. It is also important to note that *COOLAIR* is not induced by SA (Yang, unpub).

Of final note is the connection between CAMTAs and CBFs. CAMTA3 and 5 are involved in the induction of CBF1 and CBF2, but not CBF2, in response to rapid drops in temperature during the day and the night (Kidokoro, Yoneda et al. 2017). The fact that the *COOLAIR* promotor could potentially bind two sets of interacting factors could have implications for very complex feedbacks within the cold acclimation pathway.

3.4.5 NTL8 may be involved in *COOLAIR* induction in response to cold

Another factor, NTL8, was found to bind conserved sequences in the *COOLAIR* promotor in vitro. Interestingly, an NVB *COOLAIR*: LUC mutant with impaired cold induction of *COOLAIR*: LUC, 2273, was identified as a dominant gain of function mutation in NTL8 (Yang and Zhao, unpub). The loss of function NTL8 mutant has no phenotype likely due to redundancy with NTL14 and NTL5.

In 2273, *COOLAIR*: LUC, endogenous proximal *COOLAIR* and VIN3 are all transcriptionally elevated under NV conditions. A similar dominant gain of function mutant in NTL8 was identified in a parallel VIN3: LUC screen to identify thermosensors involved in VIN3 induction in response to vernalisation (Zhao, unpub). NTL8 could be part of a shared thermosensing pathway that affects *COOLAIR* and VIN3 induction in response to prolonged cold, a pathway that therefore affects both the transcriptional shutdown and nucleation of epigenetic marks to silence *FLC*.

3.5 Future Experiments

3.5.1 Mutation of cis binding sites in *COOLAIR*: LUC

Given the presence of potential cis binding sites in the *COOLAIR* promotor it would be informative to investigate *COOLAIR*: LUC induction when these sites are mutated. This could reveal whether these binding sites are necessary for *COOLAIR*: LUC induction.

Currently, five different versions of the *COOLAIR*: LUC transgene have been created by mutation of the various binding sites (Figure 3-7). In *COOLAIR*: LUC 1, both CBF sites have been mutated from a CCGAC core motifs to an irrelevant TACGT sequence (Li, Qin et al. 2015). In *COOLAIR*: LUC 2, the CAMTA binding site shown to be conserved across four Brassicaceae species has been mutated from CGCGT to ATGCT (Walley, Coughlan et al. 2007). In *COOLAIR*: LUC 3, the conserved CAMTA site was mutated as in *COOLAIR*: LUC 2 but additionally a CAMTA consensus sequence present just upstream of *COOLAIR* transcription start site was also mutated in case this site is functional in Arabidopsis thaliana. This site is not conserved across the Brassicaceae. In *COOLAIR*: LUC 4, both CBFs sites are mutated alongside the conserved CAMTA site whilst in *COOLAIR*: LUC 5 both CBF and CAMTA sites are mutated as described above.

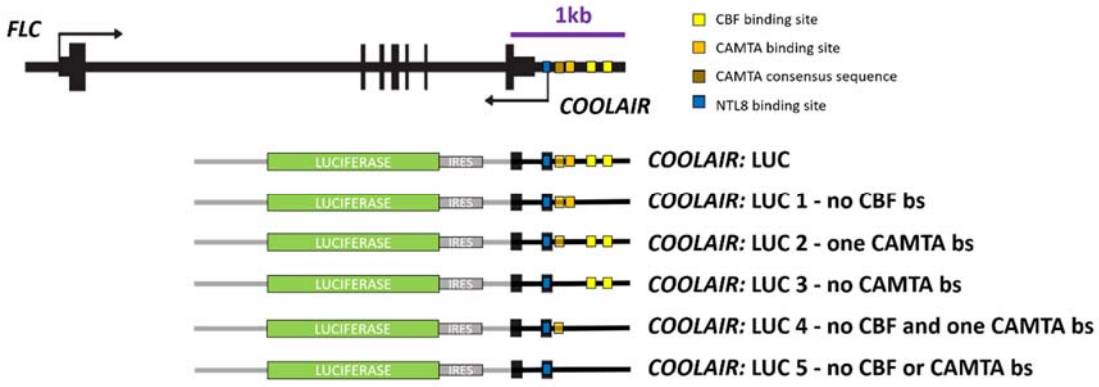


Figure 3-7 – Mutation of cis binding sites in *COOLAIR*: LUC constructs A series of mutations have been made in the *COOLAIR* promotor of the *COOLAIR*: LUC reporter construct to allow investigation if the role of these binding sites in *COOLAIR*: LUC induction in response to cold. *COOLAIR*: LUC contains the WT sequence of the *COOLAIR* promotor. *COOLAIR*: LUC 1 has the CBF binding site motifs replaced. *COOLAIR*: LUC 2 has the conserved CAMTA binding site motif replaced whilst *COOLAIR*: LUC 3 has another CAMTA consensus sequence mutated in addition to the conserved CAMTA binding site. *COOLAIR*: LUC 4 and 5 have the CBF binding site motifs replaced in addition to the one or both CAMTA consensus sequences respectively.

The five constructs were created by synthesising 323bp long DNA segments, each containing different versions of the *COOLAIR*: LUC promotor, and ligating them into the *COOLAIR*: LUC construct by restriction enzyme digest and an In – Fusion reaction (Takara). The new constructs were sequenced across the gene to confirm correct creation of the five different constructs.

All constructs are ready to be transformed into Arabidopsis thaliana plants. Single copy lines can be characterised and luciferase expression checked in the resultant generations under NV and V conditions. Multiple independent lines will have to be screened and average luciferase expression across these lines calculated as insertion sites will differ between each construct.

The NTL8 site may also be mutated in the future though given its location within an exon of *COOLAIR*, it may be difficult to mutate without introducing changes to the *COOLAIR* lncRNA itself.

4 Dissection of *COOLAIR* induction in the identified mutants

4.1 Background

It is not clear how *COOLAIR* is induced in response to weeks of cold. A forward genetic mutant screen identified a group of mutants that exhibited high *COOLAIR*: LUC levels before cold exposure and impaired induction in response to cold temperature (Chapter 3). One of these mutants, 2265, was identified as a loss of function mutation in ARP6, a core component of the SWR1 histone remodelling complex that deposits H2A.Z in chromatin. ARP6 has long been known to influence *FLC* expression (Choi, Kim et al. 2005; Martin-Trillo, Lazaro et al. 2006; Deal, Topp et al. 2007) and play a role in thermosensing (Kumar and Wigge, 2010) but it has never been linked to influencing the induction of *COOLAIR*, as identified by the forward genetic screen.

A DAP-seq database identified certain cold induced factors that bind the *COOLAIR* promotor in vitro (Chapter 3). The three redundant CBF factors (CBF1, CBF2 and CBF3) have been categorised as 'master regulators' of the cold acclimation pathway and are required for increased freezing tolerance after previous exposure to cold temperature. (Jaglo-Ottosen, Gilmour et al. 1998; Gilmour, Sebolt et al. 2000; Chinnusamy, Ohta et al. 2003; Gilmour, Fowler et al. 2004; Novillo, Alonso et al. 2004).

A role for CBF1 in vernalisation has previously been rejected (Liu, Gilmour et al. 2002) based on CBF1 overexpressing lines that did not have an altered vernalisation response. This study focused primarily on VIN3 induction and did not consider how the induction of *COOLAIR* is affected by overexpression of CBF1. In CBF1, 2 or 3 overexpressing lines, *FLC* levels before vernalisation are higher and, whilst vernalisation reduces *FLC* levels as in WT plants, intermittent cold treatments no longer affect *FLC* expression (Seo, Lee et al. 2009).

It is unknown if CBF expression patterns could correlate with induction of *COOLAIR* over weeks of cold given that few studies have tracked CBF expression over more than 24 hours (Vogel, Zarka et al. 2005; Lee, Fleming et al. 2009).

The experiments presented below were all carried out by myself. The arp6 (2265) F3 lines are progeny of an F2 plant created by Hongchun Yang by crossing the identified 2265 mutant with the

CTL progenitor line. These lines are homozygous for the arp6 (2265) mutation and *COOLAIR*:*LUC* but may contain different background EMS mutations.

4.2 Aim

Investigate the role of CBF1, 2 and 3 and ARP6 in *COOLAIR* induction and *FLC* downregulation in response to weeks of cold.

4.3 Results

4.3.1 *COOLAIR* was not induced by cold in a *cbfs* triple mutant but was induced in a *cbf1,3* double mutant

Total *COOLAIR* was increased three-fold in Col-0 in response to three weeks of cold exposure. This induction after three weeks of cold was lost in the *cbfs* triple mutant (Figure 4-1A). In the cbf1,3 double mutant, induction of total *COOLAIR* after three weeks was similar to Col-0. The induction of total *COOLAIR* in Col-0 and cbf1,3 occurred in response to one week of cold with little increase in total *COOLAIR* levels after one week. In *cbfs,* induction after one week was decreased and there was no further induction observed after two or three weeks of cold.

Spliced *FLC* and unspliced *FLC* levels decrease in response to weeks of cold in Col-0 (Figure 4-1B, 4-1C). The downregulation in spliced *FLC* is comparable to downregulation observed in *cbf1,3* or *cbfs* whilst downregulation of unspliced *FLC* is slightly impaired in *cbf1,3* and *cbfs*

4.3.2 The *cbfs* triple mutant had altered *FLC* and total *COOLAIR* levels before cold

Total *COOLAIR* levels were 6-fold reduced in *cbfs* relative to Col-0 in non-vernalisation (NV) conditions before exposure to cold, at a time when CBF levels are low (Figure 4-1A). This was not statistically significant as Tukey post hoc testing revealed that the significant difference (ANOVA, $p<0.05$) in total *COOLAIR* levels at NV was a result of the difference between *cbf1,3* and *cbfs*. At all vernalisation timepoints however the total *COOLAIR* level was significantly different from all vernalisation timepoints however the total COOLAIR level was significantly different from Col-0 levels (ANOVA, $p<0.05$, Tukey post hoc test). Spliced *FLC* levels were 3-fold reduced in *cbfs* relative to Col-0, a pattern that was replicated by unspliced *FLC* levels (Figure 4-1B, 4-1C). On a whole plant level, *FLC* and total *COOLAIR* levels are known to correlate in different mutant backgrounds, in the

ndx1-1 mutant total *COOLAIR* levels were increased and *FLC* levels were also increased as a result (Sun et al., 2013).

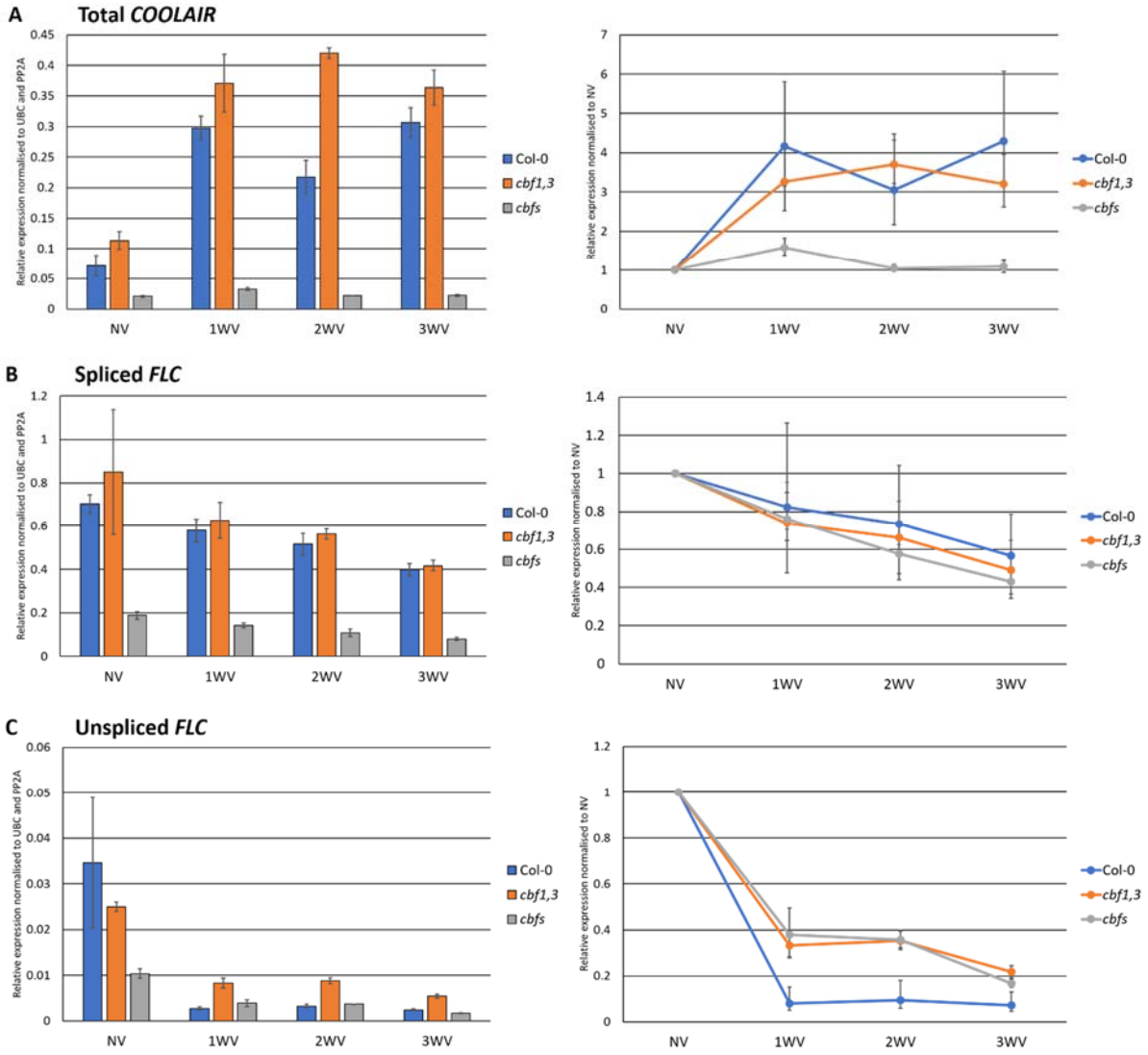


Figure 4-1 Expression of *COOLAIR* and *FLC* in *cbf1,3* and *cbfs* Expression, as measured by RT-qPCR using gene specific primers, of total *COOLAIR* (A), spliced *FLC* (B) and unspliced *FLC* (C) in non-vernalisation conditions (NV) and after one, two or three weeks of vernalisation conditions. Values presented are the means of 2-3 biological replicates and error bars represent s.e.m. ANOVA tests were carried out comparing all three genotypes at each timepoint. At all timepoints there were significant differences ($p<0.05$) in total COOLAIR expression, a Tukey post hoc test revealed that these differences were between cbf1,3 and cbfs at all time points, cbfs and Col-0 at 1WV, 2WV and 3WV time points and between Col-0 and cbf1,3 at the 2WV time point. There were significant differences ($p<0.05$) in spliced FLC expression at 1WV, 2WV and 3WV as a result of cbfs differing from Col-0 and cbf1,3 (Tukey post hoc Test). There were also significant differences ($p<0.05$) in unspliced FLC expression at 1WV, 2WV and 3WV but this was as a result of cbf1,3 differing from Col-0 and cbfs (Tukey post hoc test). The line graphs show *COOLAIR* induction or *FLC* downregulation by normalisation of expression values to NV conditions. Statistical analysis not performed on values normalised to NV.

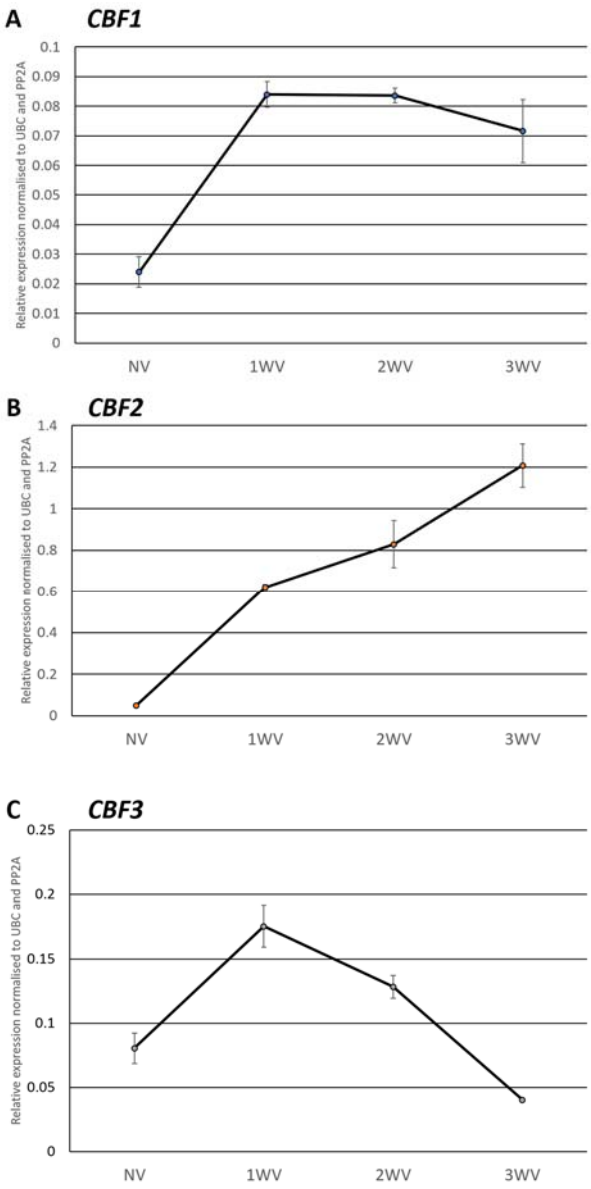


Figure 4-2 Expression of CBF1, CBF2 and CBF3 in Col-0 Expression, as measured by RT-qPCR using oligo(dT), of *CBF1* (A), CBF2 (B) and CBF3 (C) in non-vernalisation conditions (NV) and after one, two or three weeks of vernalisation conditions. Values presented are the means of 3 biological replicates and error bars represent s.e.m. Statistical analysis not carried out as samples at each timepoint are not independent from each other.

4.3.3 The CBFs were expressed differently in response to weeks of cold

The CBFs are transcriptionally induced by short term cold temperatures (Vogel, Zarka, Van Buskirk, Fowler, & Thomashow, 2005) however the expression of CBFs in response to vernalisation are not well understood. In Col-0 CBF1 was transcriptionally induced 4-fold after one week of cold relative to NV and plateaued after two or three weeks of cold (Figure 4-2A). CBF2 was induced 12-fold by one week of cold and induced further to 20-fold by three weeks of cold. (Figure 4-2B). CBF3 was induced two-fold by one week of cold and drops in level after two and three weeks of cold to an absolute level below the starting NV level (Figure 4-2C). It is unclear how much the CBF factors were induced by the initial hours of cold exposure in this experiment, so it is difficult to compare the strength of CBF induction by longer term cold relative to the induction that is usually reported.

4.3.4 *COOLAIR*: LUC induction was impaired in *arp6* (2265)

In the CTL line, *COOLAIR*: LUC was induced three-fold in response to three weeks of vernalisation treatment (Figure 4-3A). Induction begins after one week of vernalisation and was linearly correlated with length of cold exposure. In three *arp6* (2265) F3 lines, which are homozygous for the *arp6* mutation and *COOLAIR*: LUC but have different background EMS mutations, this induction was impaired resulting in a two-fold increase of *COOLAIR: LUC* between NV and 3WV. The linear response of *COOLAIR: LUC* was also altered with almost no induction between the NV and 2WV timepoint.

COOLAIR: LUC levels were two-fold higher in 2265 compared to the CTL line at NV, prior to cold exposure (Figure 4-3A), a significant difference (ANOVA, $p<0.05$) shown between the CTL line and each arp6 2265 F3 line (Tukey post hoc test). This difference was lost at 2WV and 3WV timepoints when absolute levels of *COOLAIR: LUC* were similar between the CTL line and *arp6* (2265) and there were no significant differences (ANOVA). This leads to the observed impaired induction of *COOLAIR: LUC* when relative expression values were normalised to the NV starting level.

4.3.5 Endogenous proximal *COOLAIR* had impaired induction despite similar starting levels in *arp6* (2265)

Endogenous spliced proximal *COOLAIR* was induced three-fold in Col FRI and the CTL line in response to 3 weeks of cold whereas in two of the three 2265 lines, this induction was reduced to 2-fold (Figure 4-3B). This paralleled the altered induction of *COOLAIR: LUC* in these mutants.

Starting levels (NV) of endogenous proximal *COOLAIR* were similar or slightly reduced in the 2265 mutant relative to Col FRI and the CTL line (Figure 4-3B), not a significant difference (ANOVA). Endogenous proximal *COOLAIR* levels remained lower than Col FRI or CTL levels throughout the period of vernalisation and were significantly different at 2WV and 3WV (ANOVA, $p<0.05$). This phenomenon did not apply to *COOLAIR: LUC* as previously mentioned where NV levels are two-fold higher.

As mentioned, *FLC* and total *COOLAIR* levels are known to correlate in different mutant backgrounds. Spliced and unspliced *FLC* levels were roughly halved in 2265 F3 lines relative to Col FRI and CTL lines at all timepoints (Figure 4-3C, 4-3D) shown by significant differences at all timepoints tested (ANOVA, $p<0.05$). Downregulation of *FLC* was similar between mutant and WT (normalization not

shown).

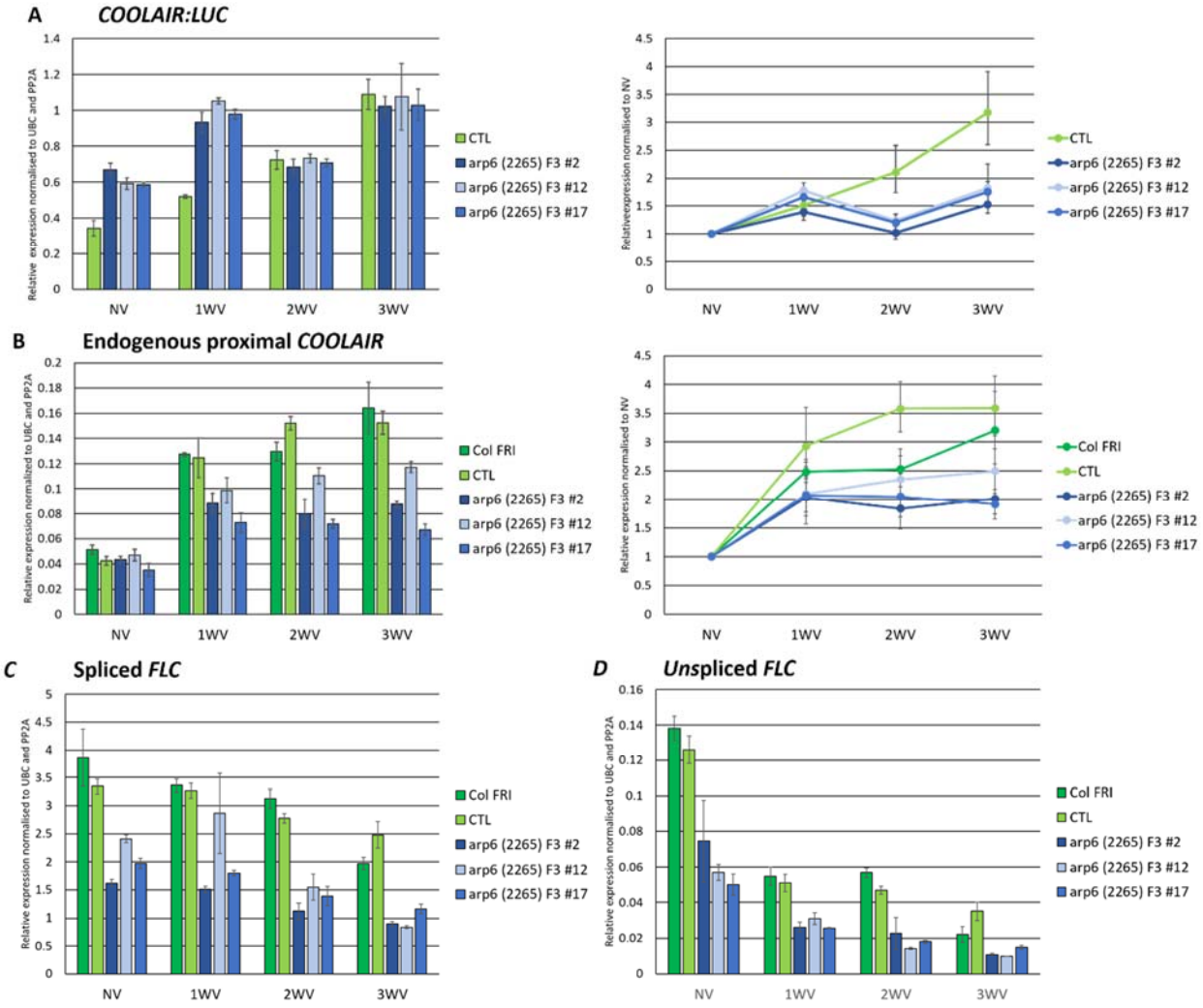


Figure 4-3 Expression of *COOLAIR: LUC*, endogenous proximal *COOLAIR* and *FLC* in *arp6* (2265) F3 lines compared to CTL and Col FRI lines Expression, as measured by RT-qPCR using gene specific primers, of *COOLAIR: LUC* (A), endogenous proximal *COOLAIR* (B), spliced *FLC* (C) and unspliced *FLC* (D) in non-vernalisation conditions (NV) and after one, two or three weeks of vernalisation conditions. Values presented are the means of 3 biological replicates and error bars represent s.e.m. Across the genotypes used, for each timepoint and each transcript an ANOVA test was carried out. For COOLAIR: LUC expression, significant differences ($p<0.05$) were found at NV and 1WV timepoints and this variation was between the CTL line and each of the F3 lines (2,12 and 17) (Tukey post hoc test). For proximal COOLAIR expression, significant differences ($p<0.05$) were found at 2WV and 3WV timepoints and this variation between was Col FRI or CTL and F3 lines 2 and 17. F3 line 12 was significantly different to the CTL line at 2WV. (Tukey post hoc test). For spliced FLC expression there were significant differences ($p<0.05$) across all timepoints though the genotypes responsible for this varied across time points (Tukey post hoc test), though all differences were between Col FRI or CTL and the arp6 2265 F3 lines. For unspliced FLC expression there were significant differences ($p<0.05$) across all timepoints though the genotypes responsible for this varied across time points (Tukey post hoc test), though all differences were between Col FRI or CTL and the arp6 2265 F3 lines. The line graphs show *COOLAIR: LUC* or endogenous proximal *COOLAIR* induction by normalisation of expression values to NV conditions. Statistical analysis not performed on values normalised to NV due to normalisation calculation.

4.3.6 *arp6* (2265) did not have elevated levels of CBF transcription factors before cold exposure

CBF transcription factors are known to be induced rapidly in response to short term cold temperature and H2A.Z gene body deposition is known to be associated with responsive genes (Coleman-Derr & Zilberman, 2012). *arp6* mutants have a range of phenotypes due to the widespread role H2A.Z has in plant development. It therefore seemed prudent to check expression of CBF factors in *arp6* (2265). It could be hypothesised that CBFs are elevated without cold exposure in the *arp6* background resulting in higher *COOLAIR*: LUC levels before cold.

In Col FRI and the CTL line, CBF1 and CBF2 were induced 10 to 30 – fold at 3WV from very low NV levels (Figure 4-4A, B). CBF1 and CBF2 were induced by each additional week of cold whilst CBF3 was induced six-fold by 1WV with less obvious further induction at two and three weeks (Figure 4-4C) CBF3 appeared to be more strongly induced by three weeks cold in the CTL line relative to Col FRI.

CBF1 and 2 levels were similar or slightly lower NV in the *arp6 (2265)* mutants relative to WT (Figure 4-4A, B), they were significantly different at 2WV and 3WV timepoints (ANOVA, $p<0.05$). This was opposite to expectation if the *COOLAIR: LUC* phenotype in *arp6* was due to elevated CBF expression. CBF3 transcripts were slightly higher but not to the levels observed at any time points in the vernalisation treatments, when proximal *COOLAIR* induction was occurring (Figure 4-4C). There was much more variability observed in CBF expression between the *arp6 (2265)* F3 lines and even between the Col FRI and CTL lines as shown by Tukey post Hoc testing. In these tests significant differences were shown to occur between Col FRI and CTL lines and between individual *arp6* (2265) F3 lines.

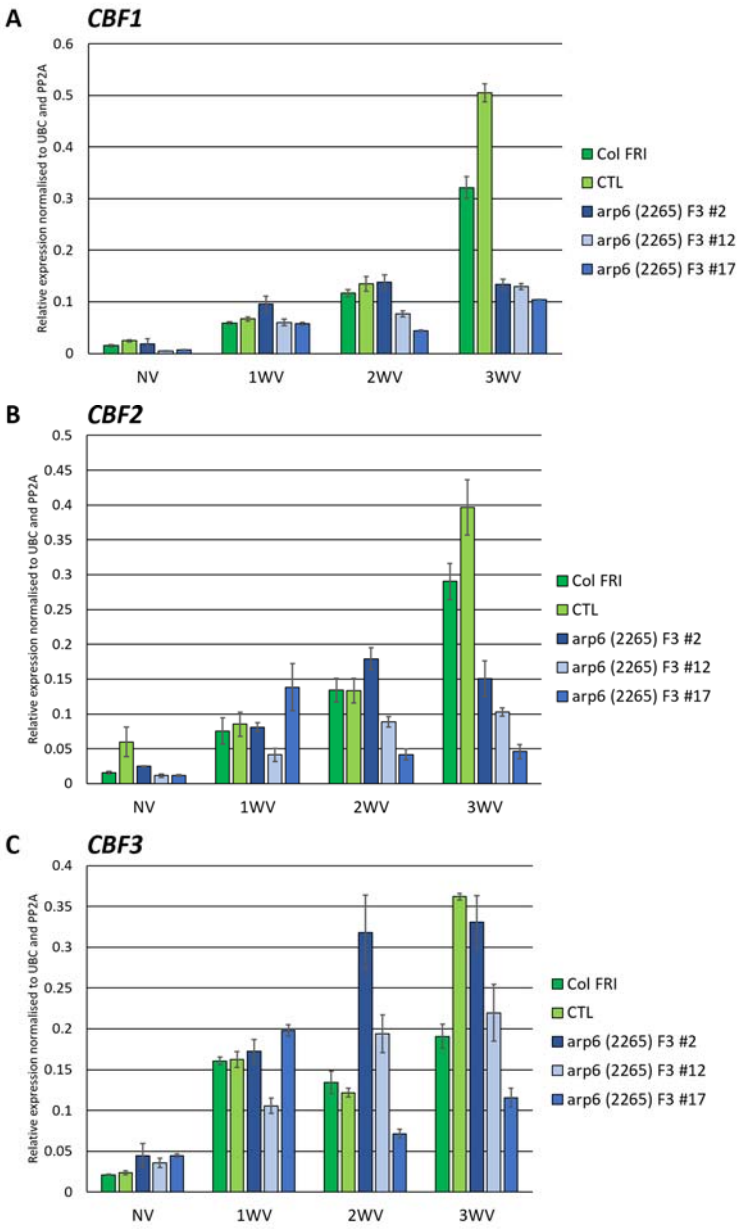


Figure 4-4 Expression of CBF1, CBF2 and CBF3 in *arp6* (2265) F3 lines compared to CTL and Col FRI lines Expression, as measured by RT-qPCR using oligo(dT), of *CBF1* (A), *CBF2* (B) and *CBF3* (C) in non-vernalisation conditions (NV) and after one, two or three weeks of vernalisation conditions. Values presented are the means of 3 biological replicates and error bars represent s.e.m. ANOVA tests were performed across all genotypes and at each timepoint for each transcript. For CBF1 expression significant differences ($p<0.05$) were found at 2WV and 3WV. Tukey post hoc testing revealed at 2WV this difference was between F3 line 17 and CTL or F3 line 2. At 3WV, this difference was between Col FRI or CTL compared to all F3 lines, but also between Col FRI and CTL. For CBF2 expression, significant differences ($p<0.05$) were found at 2WV and 3WV. Tukey post hoc testing revealed this difference was between F3 lines and CTL or Col FRI and also between some individual F3 lines. For CBF3 expression significant differences ($p<0.05$) were found at 1WV, 2WV and 3WV timepoints. Tukey post hoc testing revealed these differences were between Col FRI and CTL, Col FRI or CTL and the F3 lines or between certain individual F3 lines.

4.4 Discussion

4.4.1 The CBFs are required for the cold-induction of total *COOLAIR* in Col-0

In a triple *cbfs* mutant, total endogenous *COOLAIR* was not induced by one, two or three weeks of vernalisation unlike in Col-0. The CBF factors induce a suite of factors required for enhanced freezing tolerance after cold acclimation however they have never been linked to induction of *COOLAIR* in response to long term cold.

In Col-0 CBF1 and CBF2 were transcriptionally induced by one week of cold and remained high for two and three weeks. This correlated with total *COOLAIR* induction in Col-0 supporting the role of CBFs in cold induction of *COOLAIR*.

FLC downregulation was not impaired in the triple mutant highlighting that total *COOLAIR* upregulation and *FLC* transcriptional downregulation are potentially separable independent processes. Col-0 is not a vernalisation-requiring genotype so it may not be reasonable to describe the *FLC* downregulation observed as a vernalisation response.

4.4.2 The CBFs influence *FLC* and total *COOLAIR* levels prior to cold exposure

Surprisingly, the *cbfs* triple mutant had reduced levels of *COOLAIR* prior to any cold exposure. This implies that the CBFs may function prior to long term cold exposure to affect *COOLAIR* levels and may be due to a low but still functional level of CBF expression NV.

FLC levels were similarly reduced at the NV time point in the *cbfs* mutant. It is not possible to ascertain whether CBFs primarily affect *FLC* or *COOLAIR* transcript levels given the complex feedback between sense and antisense associated with the locus. CBF overexpression lines in the Ws background have elevated levels of *FLC* NV so the loss of function phenotype observed is consistent with the literature (Seo et al., 2009).

4.4.3 *arp6* is epistatic to cold temperature on *COOLAIR: LUC* and proximal *COOLAIR* expression

In *arp6* (2265), *COOLAIR: LUC* was elevated under NV conditions and induction by cold exposure in impaired. This could be due to saturation of *COOLAIR: LUC* levels that cannot be induced any further

due to physical limitations. In other words, *COOLAIR: LUC* is being maximally expressed as a result of the *arp6* mutation and cold at 1WV. This may be rejected given that expression levels show that after two weeks of cold *COOLAIR: LUC* levels have actually been reduced from 1WV. This behaviour of *COOLAIR: LUC* could therefore show that cold and ARP6 work in the same pathway to induce *COOLAIR: LUC*. ARP6 represses COOLAIR: LUC expression but exposure to weeks of cold represses ARP6 or factors downstream of ARP6 leading to increased *COOLAIR: LUC* expression. In *arp6*, repression of *COOLAIR: LUC* is lost therefore cold has no further effect on *COOLAIR: LUC* expression. Induction after one week still occurs as a result of other cold affected factors. Endogenous proximal *COOLAIR* is similarly induced less by cold treatment despite not being elevated at NV.

4.4.4 Complex feedback explains why proximal *COOLAIR* is not elevated NV in *arp6*

In *arp6 (2265)* lines, *COOLAIR: LUC* is elevated at NV conditions however endogenous proximal *COOLAIR* is not. Promotion of proximal *COOLAIR*, due to *arp6*, could secondarily silence the locus resulting in the observed reduced expression of *FLC* and proximal *COOLAIR*. This effect has been observed in *prp8* and *cdkc2* mutants (Marquardt et al., 2014; Wang, Wu, Raitskin, Sun, & Dean, 2014). In these cases, the mutation results in loss of proximal *COOLAIR* expression (a loss of *COOLAIR: LUC* expression) however this causes de-repression of the endogenous locus leading to the observed increased expression of *FLC* and endogenous proximal *COOLAIR*. Alternatively, *arp6* may independently affect both *COOLAIR* and *FLC* expression, given that H2A.Z is found at both the 5' and 3' ends of the *FLC* gene, and the resulting changes in expression observed are due to the complex interplay between *COOLAIR* and *FLC*.

4.4.5 CBF1 and CBF2 may be induced less by cold in *arp6*

Given the cold induction associated with the CBF factors and the lack of induction of total *COOLAIR* observed in the loss of function triple mutant, it is possible that the *COOLAIR* phenotype observed in *arp6* is the result of mis-regulated CBF factors. The CBF factors are not however elevated in *arp6* (2265) therefore the elevated basal level of *COOLAIR*: LUC is not due to increased expression of the CBF factors. Interestingly, CBF1 and CBF2 do not appear to be as induced by three weeks of cold in the ARP6 mutant. This correlates with reduced induction of *COOLAIR:LUC* and proximal *COOLAIR* at three weeks of cold in *arp6*.

4.4.6 Model: Cold induces *COOLAIR* through the induction of CBF factors and by repressing an ARP6-associated pathway

Overall, the CBFs activate *COOLAIR* expression under a range of conditions. When the *FLC* gene loop is present (NV), CBFs promote *COOLAIR* expression and the locus is activated to produce *FLC* and total *COOLAIR* to a high level, relative to the *cbfs* mutant. In response to cold, the CBFs are induced, presumably in the short term but also in response to weeks of cold resulting in *COOLAIR* induction. In response to cold the gene loop is broken, as is the complex feedback between *FLC* and *COOLAIR*, so *FLC* does not increase and is in fact downregulated in a potentially independent process (Figure 4-5).

Meanwhile, ARP6 represses *COOLAIR* expression NV. At the endogenous locus, repression of proximal *COOLAIR* or independent functions of ARP6 on *FLC* leads to high expression of *FLC* and endogenous *COOLAIR*, relative to the *arp6* mutant. Cold represses the ARP6-associated pathway, resulting in increased *COOLAIR*. Additionally, ARP6 may be required for full induction of CBF1 and CBF2 in response to three weeks of cold (Figure 4-5).

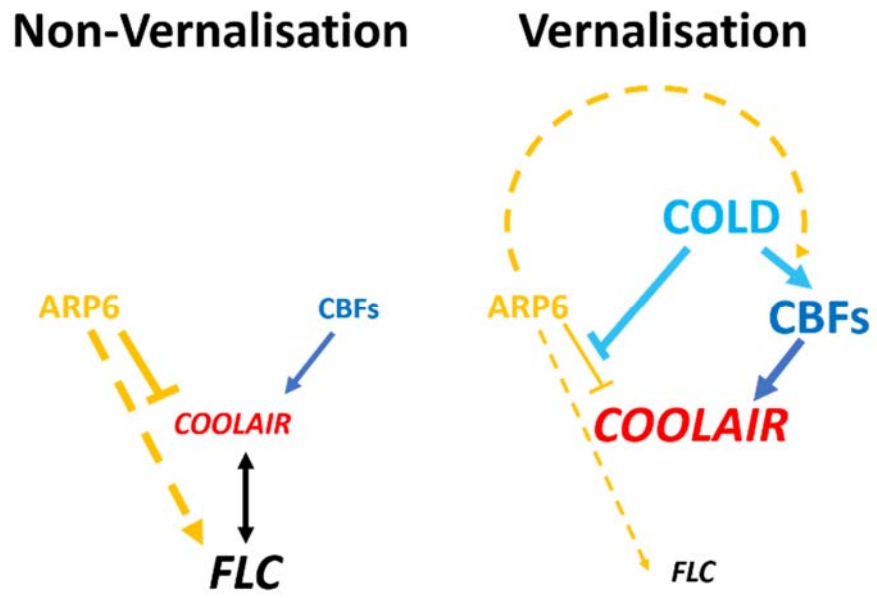


Figure 4-5 Model showing ARP6 and CBF1,2,3 regulating the induction of *COOLAIR* in response to weeks of cold In NV conditions, CBFs activate *COOLAIR* expression and ARP6 represses *COOLAIR* expression (and may independently promote *FLC* expression). *COOLAIR* and *FLC* display complex feedback properties as a result of the gene loop. In response to vernalisation conditions, this feedback is broken. The CBFs are transcriptionally induced by cold, perhaps requiring ARP6 for full induction, and the ARP6-associated upstream of *COOLAIR* is repressed by cold.

4.5 Future Experiments

4.5.1 Investigate the role of CBFs on induction of *COOLAIR* in a FRI containing line and on induction of *COOLAIR: LUC*

The *cbfs* triple mutant has implicated a role for the CBF factors in inducing *COOLAIR* in response to weeks of cold. The Col-0 background genotype is not however a vernalisation-requiring genotype raising questions of the relevance of this finding. The *cbfs* mutant has been crossed to Col FRI meaning *COOLAIR* induction, in a *cbfs* triple mutant, can be tested in a vernalisation-requiring line. The *cbfs* triple mutant has also been crossed to the *COOLAIR: LUC* CTL line so that the direct effect of *cbfs* on *COOLAIR*, independent of *FLC*, can be determined.

4.5.2 Investigate induction of total endogenous *COOLAIR* in *arp6*, *hta9 hta11* and *ino80*

ARP6, as part of the SWR1 remodelling complex, affects the widespread expression of many *Arabidopsis* genes by affecting H2A.Z localisation within chromatin. To confirm the mechanism through which ARP6 affects *COOLAIR* it is important to confirm that the effect is mediated through H2A.Z by checking *COOLAIR* induction in a double H2A.Z mutant, *hta9 hta11* (March-Diaz et al., 2008).

H2A.Z is found at the 5' and 3' end of *FLC*. In *arp6*, H2A.Z is lost from both the 5' and 3' end of *FLC* and this means it is impossible to dissect how endogenous *COOLAIR* behaves in response to loss of H2A.Z at the 3' end independently of loss of H2A.Z from the 5' end. The *COOLAIR*: LUC *arp6 (2265*) phenotype theoretically represents the result of loss of H2A.Z from only the 3' end of *FLC*. Interestingly in another mutant, *ino80-5*, H2A.Z is lost only from the 3' end of *FLC* and not the 5' end (Zhang et al., 2015). It would therefore be interesting to measure endogenous total *COOLAIR* induction in *ino80,* which recreates the effect of *arp6* at the 3' end but not the 5' end of *FLC*. INO80 is part of another chromatin remodelling complex that redistributes H2A.Z in mammals and yeast (Gerhold & Gasser, 2014; Knezevic, Gonzalez-Medina, Gaspa, Hidalgo, & Ayte, 2018).

4.5.3 Map H2A.Z at *COOLAIR: LUC* and the endogenous *FLC* locus in response to cold

As *arp6* is not additive to exposure to cold, it can be hypothesised that *arp6* and cold affect *COOLAIR* expression through a similar mechanism. This could manifest itself as a loss of H2A.Z at *COOLAIR* in response to cold. H2A.Z has previously been shown to be displaced from chromatin in response to

warm temperature and *arp6* plants display a constitutive warm temperature transcriptome (Kumar & Wigge, 2010). It is not known how H2A.Z dynamics alter in the cold. H2A.Z distribution can be analysed at the endogenous *FLC* locus in HTA9-GFP FRI lines using Chromatin Immunoprecipitation (ChIP). Exposure of these plants to vernalisation conditions will allow H2A.Z distribution changes in response to cold to be determined.

It is not known whether H2A.Z localises similarly in *COOLAIR: LUC* as it does at the endogenous *COOLAIR* promotor. Similar localisation of H2A.Z at the endogenous locus and the transgene would support the role of H2A.Z in regulating *COOLAIR* expression levels. Given the sequence identity between *COOLAIR*: LUC and the *COOLAIR* promotor, a line that has the full endogenous *FLC* locus deleted must be crossed to a HTA9-GFP *COOLAIR*: LUC line to allow mapping of H2A.Z at *COOLAIR*: LUC by ChIP.

5 Investigation of *COOLAIR* induction in response to different temperature regimes

5.1 Background

COOLAIR transcripts were first identified when it was found that they are induced in response to two weeks of constant 5°C vernalisation conditions concomitant with downregulation of sense *FLC* transcripts (Swiezewski et al., 2009). Since this discovery, it has been shown that, whilst total *COOLAIR* is induced by weeks of constant 5°C, total *COOLAIR* is not comparably induced by constant 12°C, 14°C or 22°C (Heckmann, unpub).

In nature temperature fluctuates across a massive range between the day and night, between days within a week and across the seasons; temperature inputs to *COOLAIR* are likely to be complex and multiple. This control would permit the plant to induce *COOLAIR* expression in response to weeks of winter cold, which would have a different temperature profile every year, and to not induce in response to a cooler day in summer or early autumn.

VIN3 expression is also induced by weeks of constant 5°C however experiments investigating VIN3 expression dynamics in response to fluctuating temperature regimes identified that VIN3 expression responds to four temperature inputs and does not simply detect 'weeks of constant 5°C'. *FLC* downregulation was shown to be controlled by two additional thermosensitive stages including a VIN3-independent stage that responds to night-time temperature (Antoniou-Kourounioti et al., 2018).

Given *COOLAIR* induction has only been studied under constant temperature regimes it is impossible to determine what the thermosensors regulating *COOLAIR* are responding to. It is initially interesting to investigate the effect of fluctuating 5°C temperatures on *COOLAIR* expression, which can inform on future experiments to precisely identify the temperature inputs involved at *COOLAIR*.

Field data in Sweden from the winter of 2016 revealed that total *COOLAIR* is induced by sub-zero temperatures (Hepworth, Antoniou-Kourounioti, Doughty, Heckmann, Berggren, Selga, Tudor, Bloomer, Cox, Collier Harris, Yates, Wu, Irwin, Säll, Holm, Howard and Dean, in prep). This *COOLAIR* expression peak dwarfed the peak observed over weeks in response to prolonged cold. This

phenomenon was replicated in cabinets programmed with an identical temperature regime across a week. Total *COOLAIR* was induced 8-fold in response to freezing temperatures peaking after eight hours from the first exposure.

In the field, temperatures dropped below freezing at multiple times though *COOLAIR* did not respond in a similar way. This could be due to the low resolution of the field experiments where *COOLAIR* expression was checked only weekly. It is therefore unclear if *COOLAIR* is upregulated in response to the first freezing spike or all subsequent spikes.

Additionally there is some evidence that COOLAIR is expressed in a diurnal pattern whereas FLC is not (Hepworth, Antoniou-Kourounioti, personal communication).

The following experiments were carried out by myself informed by previous data gathered by the lab.

5.2 Aims

COOLAIR is known to increase in response to two weeks of prolonged cold (constant 5°C) and in response to short-term sub-zero temperatures. Investigating total *COOLAIR* induction in response to sub-zero conditions and fluctuating 5°C conditions could reveal interesting expression patterns that could, in the future, lead to a temperature input model comparable to the one created for VIN3 expression.

5.3 Results

The results presented below may form part of a publication currently in preparation: Hepworth, Antoniou-Kourounioti, Doughty, Heckmann, Berggren, Selga, Tudor, Bloomer, Cox, Collier Harris, Yates, Wu, Irwin, Säll, Holm, Howard and Dean.

5.3.1 Total *COOLAIR* was induced by a freezing spike

Total *COOLAIR* is induced by sub-zero temperatures in the field however *COOLAIR* dynamics in response to a change from constant 5°C to constant sub-zero temperatures has not specifically been

tested. This experiment would rigorously determine if sub-zero temperatures specifically cause the massive induction of total *COOLAIR* observed in the field, as opposed to a large drop in temperature.

To test this, *Arabidopsis thaliana* plants were pre-grown for ten days under NV conditions, transferred to constant 5˚C vernalisation conditions for four days, allowing the plants to acclimatise, before either remaining in constant 5°C vernalisation conditions or being transferred to sub-zero vernalisation conditions for 32 hours. Samples were taken after pre-growth (NV), after cold acclimation and after 4,8,12,24 or 32 hours of sub-zero temperatures (solid lines) or continued constant 5°C vernalisation (dashed lines).

Proximal *COOLAIR* was induced after exposure to eight hours of sub-zero temperature compared to exposure to continued vernalisation conditions (Figure 5-1A). In Col-0, proximal *COOLAIR* was induced six-fold from NV starting level by the four-day cold acclimation. Further exposure to vernalisation conditions did not alter proximal *COOLAIR* expression however exposure to sub-zero conditions led to a further three to four-fold increase in proximal *COOLAIR* expression over 32 hours (Figure 5-1A, D). Therefore, short - term freezing induced proximal COOLAIR expression beyond the level induced by vernalisation temperatures.

5.3.2 *cbfs* did not affect the freezing induction of *COOLAIR*

Given that CBF factors have been implicated in the induction of *COOLAIR* in response to vernalisation conditions (Chapter 4), it is interesting to investigate the role of CBFs in the sub-zero induction of *COOLAIR*.

As previously shown, proximal *COOLAIR* levels NV were slightly reduced in the *cbfs* triple mutant and induction of proximal *COOLAIR* in response to four days of cold acclimation was halved compared to Col-0, induced only three-fold (Figure 5-1B). Proximal *COOLAIR* levels after sub-zero temperatures did not reach the levels of Col-0 however proximal *COOLAIR* was still induced relative to a continued vernalisation treatment (Figure 5-1B). When expression levels were normalised to account for the different starting levels of proximal *COOLAIR*, after the four days of cold acclimation), *COOLAIR* was induced by sub-zero temperatures similarly in the *cbfs* mutant compared to Col-0 (Figure 5-1D).

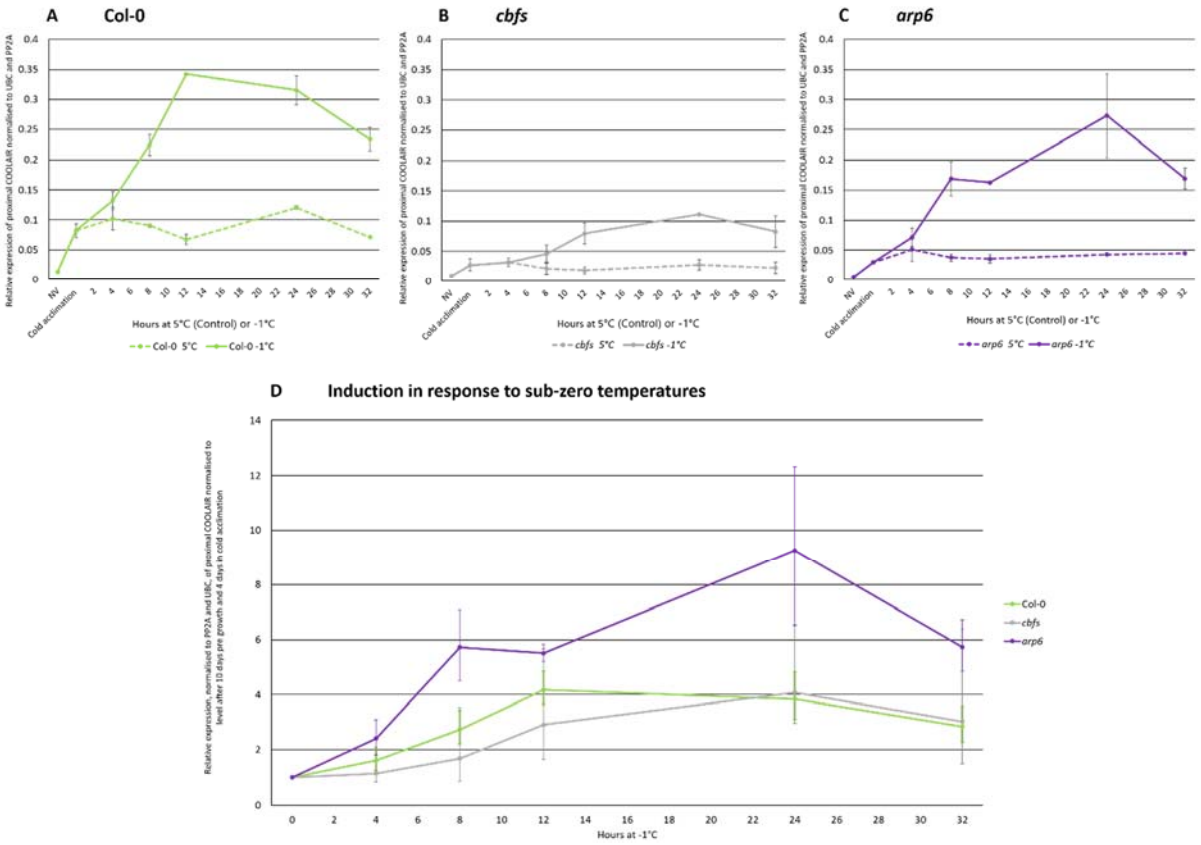


Figure 5-1 – *COOLAIR* is induced more by freezing compared to vernalisation conditions
Proximal *COOLAIR* expression normalised to UBC and PP2A after 10 days pre-growth NV, 4 days cold acclimation under vernalisation conditions and after hours spent in freezing conditions (solid line) or after continued hours spent under vernalisation conditions (dashed lines) in A) Col-0, B) *cbfs* and C) *arp6-1*. All points are the mean of one to two biological replicates. Error bars represent s.e.m. Given the lack of replicates at each time point statistical analysis has not be carried out. D) Proximal *COOLAIR* expression in response to hours of freezing normalised to the starting level of proximal *COOLAIR* after pre-growth and 4 days cold acclimation.

5.3.3 *arp6-1* increased induction of *COOLAIR* in response to freezing

ARP6 has also been implicated in the cold temperature dependent induction of proximal *COOLAIR* likely by repressing *COOLAIR* expression under NV conditions and then itself, or downstream events, being repressed under vernalisation conditions. *arp6-1* mutants were therefore tested in the freezing experiment to investigate the role of ARP6 in the freezing induction of proximal *COOLAIR*.

As expected, proximal *COOLAIR* was expressed to less than half the level of Col-0 in NV conditions but induction of proximal *COOLAIR* in response to four days of cold acclimation was similar to Col-0 (Figure 5-1C). Proximal *COOLAIR* was induced by sub-zero temperatures to similar levels to Col-0 too. Interestingly, proximal *COOLAIR* appeared to be induced slightly more in *arp6-1* than Col-0 in

response to sub-zero temperatures, increasing between six and nine-fold compared to four-fold in Col-0 (Figure 5-1D).

5.3.4 Testing *FLC* downregulation and *COOLAIR* induction in constant and fluctuating 5˚C conditions

COOLAIR induction in response to cold temperature has only rigorously been tested at constant temperatures whilst *FLC* downregulation has been tested in various temperature regimes but only in later phases of vernalisation when VIN3-dependent silencing is ongoing. To investigate the temperature inputs to *COOLAIR* induction more closely, plants were vernalised, after 10 days NV pre-growth, for two weeks at constant 5˚C or one of two fluctuating 5˚C regimes (Figure 5-2A). One of the fluctuating regimes ranges from 3˚C to 9˚C (Fluctuating 5˚C (3˚C)) across a day whereas the other ranged from -1˚C to 12˚C on a day (Fluctuating 5˚C (-1˚C)) but the average temperature of both regimes across 24 hours was 5˚C (Figure 5-2B). It was important to include a fluctuating regime that did not approach freezing conditions given total *COOLAIR* is known to be induced by short-term sub-zero temperatures. After one night of vernalisation (1DV) and after two weeks of vernalisation (2WV), six samples were taken across a 24-hour period (Figure 5- 2A, B). NV samples were taken at the same time as the 1DV samples (Figure 5-2A).

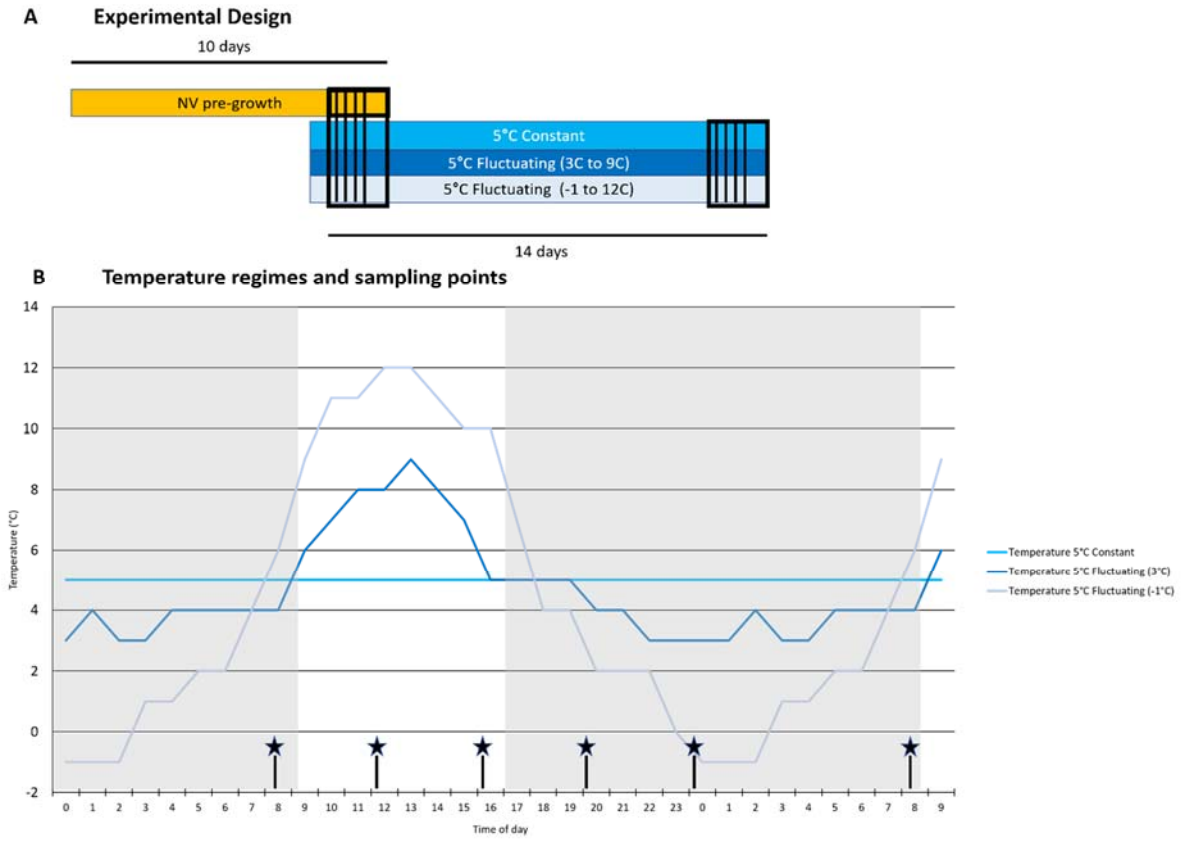


Figure 5-2 Constant 5°C and Fluctuating 5°C Experimental Design A) Plants were grown for 10 days under NV conditions. Some plants were then moved at 5pm to one of three different vernalisation treatments (Constant 5°C, fluctuating 5°C (3°C) and fluctuating 5°C (-1°C)). NV samples and 1DV samples were taken the following day at 8am, 12pm, 4pm, 8pm, 12am and then the day after at 8am. 2WV samples were taken across the day after two weeks under vernalisation conditions. B) The daily temperature profiles of each regime Constant 5°C (Bright blue), Fluctuating 5°C (3°C) (Dark blue) and fluctuating 5°C (-1°C) (Pale blue). The starred lines represent the time of day of sampling after one day of vernalisation (1DV) or after two weeks of vernalisation (2WV). The grey blocks on the graph represent night time (16 hours) and the white background represents day time (8 hours).

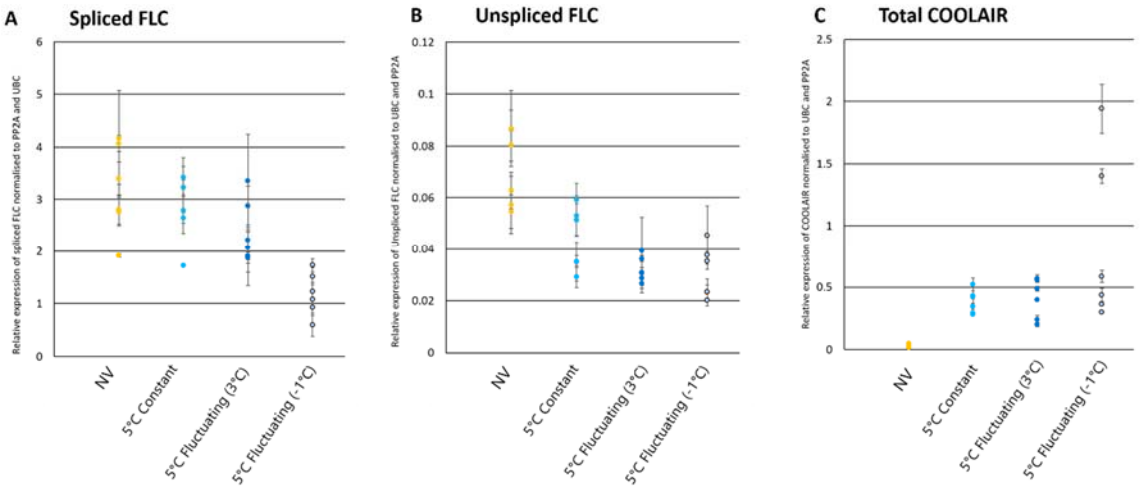


Figure 5-3 Expression analysis of *FLC* and *COOLAIR* after two weeks of vernalisation in constant and fluctuating regimes For each regime, six samples (represented by one data point) were taken throughout the day. Each data point is the mean of three biological replicates. Error bars are s.e.m. qPCR values normalised to *UBC* and *PP2A* control genes. A) Spliced *FLC* B) Unspliced *FLC* and C) Total *COOLAIR*. Comparing expression values across the three different cold temperature regimes revealed a significant difference (ANOVA, $p<0.001$) in spliced *FLC* levels but not unspliced FLC levels. This difference was between the 5°C fluctuating regime (-1°C) compared to the other two regimes (Tukey post hoc test). Given the obvious outliers statistical tests were not performed for total *COOLAIR* expression.

5.3.5 Unspliced *FLC* was downregulated similarly by all temperature conditions

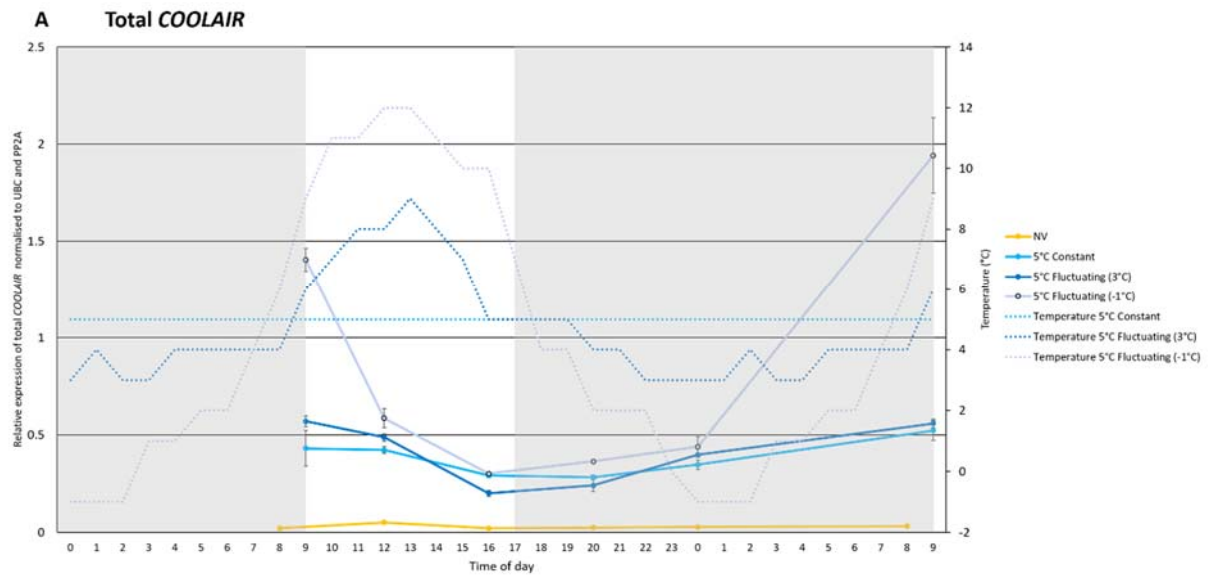
Unspliced *FLC* is an approximate measure of transcriptional activity of the *FLC* locus and can provide a readout for the degree of transcriptional downregulation that occurs during the initial phase of vernalisation. Spliced *FLC* has previously been shown to have a long half-life and therefore could be left over from a transcription event that happened prior to cold exposure (Csorba et al., 2014).

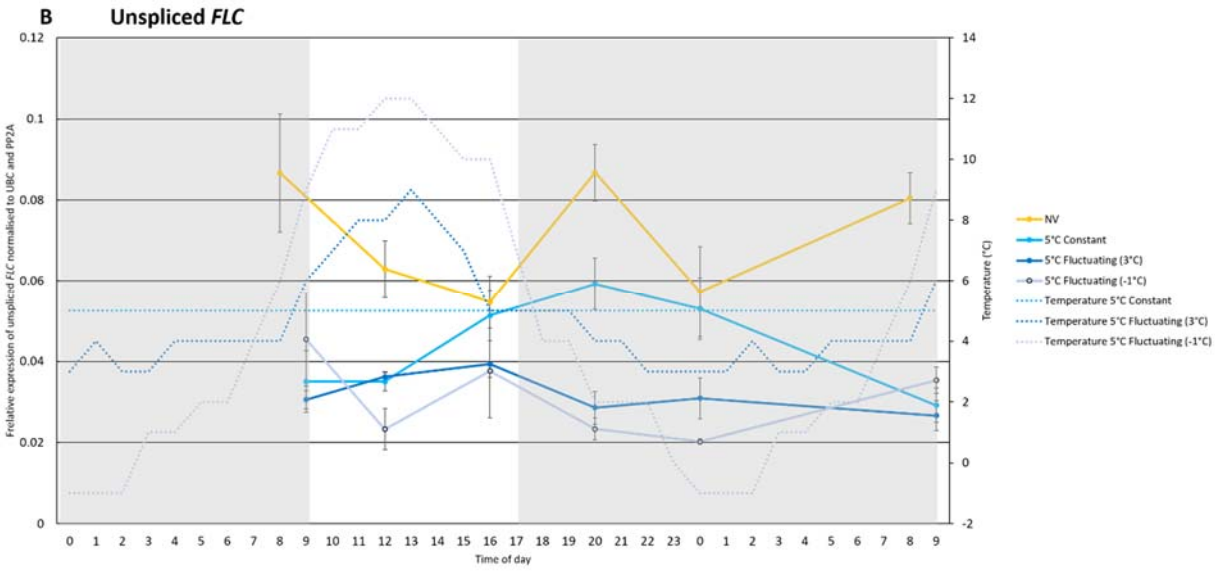
After two weeks of constant 5°C, unspliced *FLC* levels were reduced ~2 – fold compared to NV (Figure 5-3B). Unspliced *FLC* was reduced similarly in both the fluctuating 5°C regimes. There were no significant differences in unspliced FLC levels between the three 5°C regimes (ANOVA). Unspliced *FLC* levels varied broadly across a day and varied considerably between biological replicates. There was no pattern to these changing expression levels across a day comparing across the different temperature regimes (Figure 5-4B).

5.3.6 Total *COOLAIR* was induced similarly by constant and fluctuating 5°C conditions but is more induced by exposure to hours of freezing

Total *COOLAIR* was induced ~12-fold by two weeks at constant 5°C. Strikingly, at most timepoints throughout the day, total *COOLAIR* levels were induced very similarly in response to both fluctuating regimes (Figure 5-3C). However, two of the timepoints in the 5°C fluctuating (-1°C) regime very obviously displayed high levels of total *COOLAIR* expression, over 50-fold the NV starting level.

These two time points corresponded to the two 08:00 samples taken at the start and end of the 24-hour sampling period. Comparing the total *COOLAIR* profile to the temperature profile revealed that the high levels of total *COOLAIR* expression observed in the 5°C fluctuating (-1C) regime occurred six to eight hours after exposure to temperatures below 0°C (Figure 5-4A). Interestingly, total *COOLAIR* levels between 12:00 midday and 00:00 midnight were very similar between all temperature regimes despite large differences in absolute temperature during these times (Figure 5-4A).


A
Total COOLAIR
Relative expression of total COOLAIR normalised to UBC and PP2A
Temperature (°C)
Time of day
NV
5°C Constant
5°C Fluctuating (3°C)
5°C Fluctuating (-1°C)
Temperature 5°C Constant
Temperature 5°C Fluctuating (3°C)
Temperature 5°C Fluctuating (-1°C)


B
Unspliced FLC
Frelative expression of unspliced FLC normalised to UBC and PP2A
Temperature (°C)
Time of day
NV
5°C Constant
5°C Fluctuating (3°C)
5°C Fluctuating (-1°C)
Temperature 5°C Constant
Temperature 5°C Fluctuating (3°C)
Temperature 5°C Fluctuating (-1°C)

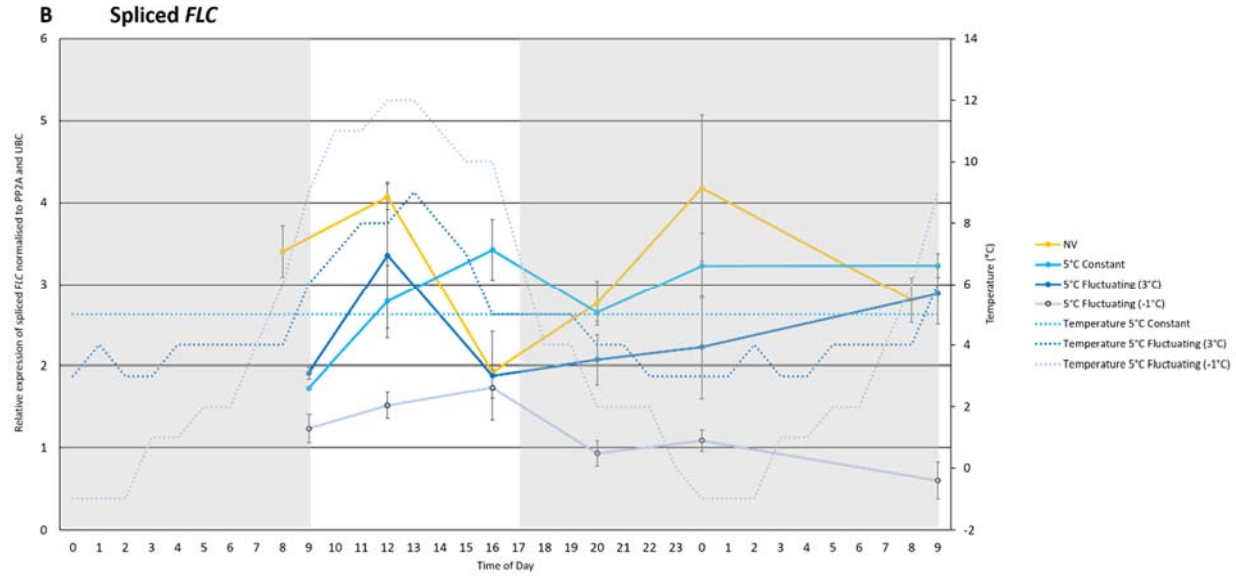

B
Spliced FLC
Relative expression of spliced FLC normalised to PP2A and UBC
Temperature (°C)
Time of Day
NV
5°C Constant
5°C Fluctuating (3°C)
5°C Fluctuating (-1°C)
Temperature 5°C Constant
Temperature 5°C Fluctuating (3°C)
Temperature 5°C Fluctuating (-1°C)

Figure 5-4 Expression profiles after two weeks of vernalisation across the day The three different temperature regimes are shown by dotted lines and night time is shown by the shaded grey background. qPCR values normalised to UBC and PP2A control genes. The six samples taken throughout the day represent the mean of three biological replicates and error present is s.e.m. NV (Yellow line), constant 5°C (Bright blue), fluctuating 5°C (3°C) (Dark blue line), fluctuating 5°C (-1°C) (Pale blue line). A) Total *COOLAIR* B) Unspliced FLC C) Spliced FLC

5.3.7 *COOLAIR* responded to each freezing spike regardless of previous exposure

The observation that total *COOLAIR* is induced by sub-zero temperatures after two weeks in a fluctuating temperature regime revealed that total *COOLAIR* can be induced by sub-zero temperatures regardless of previous exposure. From the field data, it had been hypothesised that only the first drop to temperatures below freezing would cause this effect. Total *COOLAIR* was induced by the fourteenth and fifteen sub-zero drops experienced after two weeks of the 5°C fluctuating (-1°C) regime. This induction is comparable to the induction observed after the first and second exposure to sub-zero temperatures after one day of vernalisation (1DV) (Figure 5-5A). At 1DV in the constant 5°C regime, total *COOLAIR* was induced 7- fold, around half of that observed after 2 weeks of constant 5C. At 1DV, between 16:00 and 00:00 there was no clear difference between total *COOLAIR* level in 5°C constant or 5°C fluctuating (-1°C) conditions however at 08:00 total *COOLAIR* was induced highly in the 5°C fluctuating (-1°C) condition after experiencing sub-zero temperatures during the night.

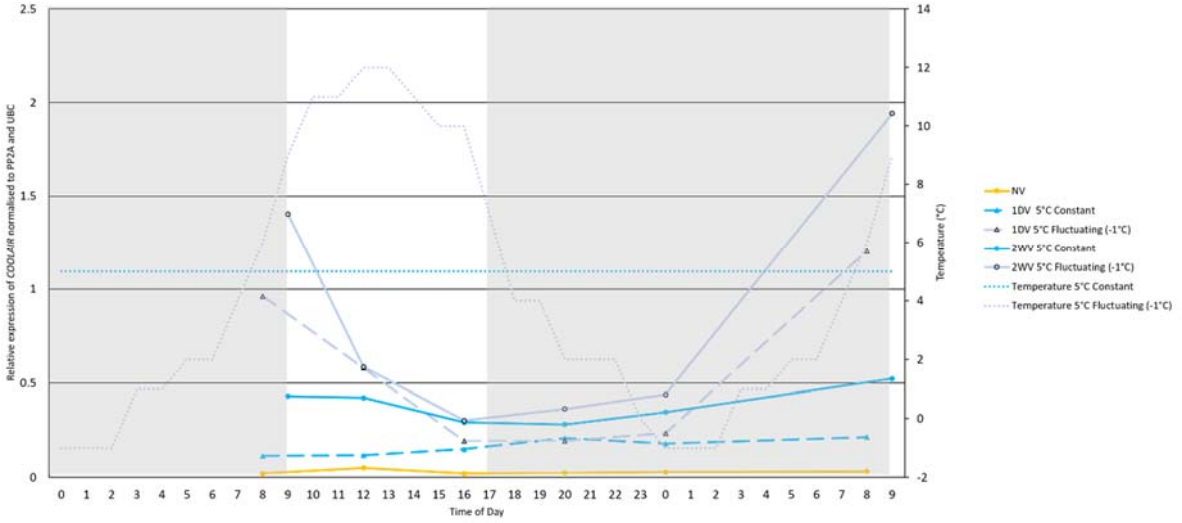


Figure 5-5 Total *COOLAIR* Expression profiles after one day and two weeks of vernalisation
Dotted lines represent the different temperature regimes. The six samples taken throughout the day after one day of vernalisation (1DV, dashed lines) or after two weeks of vernalisation (2WV, solid lines) represent he mean of three biological replicates. Error shown is s.e.m. NV (Yellow line), constant 5°C (Bright blue), fluctuating 5°C (-1°C) (Pale blue line).

5.3.8 Spliced *FLC* was downregulated more by the 5°C fluctuating regime (-1°C)

Spliced *FLC* was hardly reduced by two weeks of 5°C constant or 5°C fluctuating (3°C) vernalisation treatments (Figure 5-3A). The 5°C fluctuating (-1°C) vernalisation treatment had a clear repressive effect on spliced *FLC* levels, reducing them between two and three-fold. These expression values were significantly lower compared to the constant 5°C or fluctuating 5°C (3°C) regimes (ANOVA, $p<0.001$, Tukey post hoc testing). Given unspliced *FLC* levels were downregulated similarly by all three temperature regimes (Figure 5-3B), it is interesting that spliced *FLC* levels did not follow this pattern. The 5°C fluctuating (-1°C) regime decoupled the correlation between unspliced and spliced *FLC* levels. Spliced *FLC* is reduced at all time-points throughout the 24-hour sampling period and did not respond to sub-zero temperatures in the manner total *COOLAIR* responds (Figure 5-4C).

5.4 Discussion

5.4.1 *COOLAIR* expression responds to at least two temperature inputs

COOLAIR is induced in response to prolonged periods of cold, two weeks with an average daily temperature of 5°C, and is induced in response to current sub-zero temperatures. These two

temperature inputs are likely to be detected by at least two thermosensors given the different temperatures and timescales they are responding to.

5.4.2 *COOLAIR* responds to sub-zero temperatures rapidly, transiently and repeatedly

Current sub-zero temperatures drastically upregulated *COOLAIR* expression relative to continued vernalisation conditions. This response occurred between four to eight hours after the first exposure to sub-zero conditions.

Expression of *COOLAIR* can remain high if sub-zero temperatures continue, for up to 32 hours tested, or can drop to a basal vernalisation level within four hours from peaking if the sub-zero temperature is removed. *COOLAIR* upregulation in response to sub-zero temperatures can therefore be transient or persist if the sub-zero temperature remains.

From the field data, it was originally hypothesised that *COOLAIR* may be induced only by the first exposure to sub-zero temperatures. This is not supported by the 5˚C fluctuating experiment where *COOLAIR* was induced to a similar level by the first, second, fourteenth and fifteenth exposure to sub-zero temperatures. *COOLAIR* therefore responds repeatedly to sub-zero temperatures.

5.4.3 ARP6, but not CBF factors, is involved in upregulation of *COOLAIR* in response to sub-zero temperatures

Though likely important for *COOLAIR* induction in response to prolonged cold, the CBF factors are not important for upregulation of *COOLAIR* in response to freezing. Coincidently, CBF factors are important for cold-acclimation dependent freezing tolerance but are not required for basal freezing tolerance (Jia et al., 2016). Additionally, the CBF signalling pathway does not appear to be as important in sub-zero acclimation compared to cold acclimation. Instead, AP2/EREBP and WRKY transcription factors may be involved in this process (Le, Engelsberger, & Hincha, 2008; Le, Pagter, & Hincha, 2015).

In *arp6-1 COOLAIR* was induced more in response to sub-zero temperatures. Given the complex role of ARP6 on endogenous *COOLAIR*, described in Chapter 4, it is difficult to hypothesise why this happens. It is likely that extreme sub-zero temperatures alter the biophysical states important for transcriptional processes and histone exchange, both of which are intimately linked to ARP6.

5.4.4 Insights into how *COOLAIR* measures two weeks of prolonged cold

Aside from samples taken after exposure to sub-zero temperatures, the expression level of *COOLAIR* differs little between temperature regimes that have a daily average temperature of 5°C despite having very different daily profiles. It is difficult from this experiment alone to determine what temperature input is being detected to cause *COOLAIR* upregulation in response to prolonged cold, however various hypothesis can be made.

COOLAIR expression is not repressed by warmer temperatures (up to 12°C) experienced during the day-time. This could mean that warm temperatures have no effect on *COOLAIR* expression or that *COOLAIR* is not sensitive to temperature during the day.

In all temperature regimes most of the night time was spent at 5°C or below. Thermosensory inputs at *COOLAIR* could only be responsive during the night-time and the level of *COOLAIR* during the day could be impacted by the temperature of the previous night or nights.

COOLAIR induction in response to prolonged cold may be the result of the plant measuring the number of hours per day spent below a certain temperature. Once the daily quota is reached, *COOLAIR* may be induced to a certain level. For example, *COOLAIR* may only be induced after experiencing 12 hours per day of temperatures below 5°C. If it receives more than this, *COOLAIR* is not induced further. All three temperature regimes would fulfil this and hence in all regimes, *COOLAIR* is induced to a similar level throughout the day.

This initial experiment will inform future experimental designs to allow the dissection of the thermosensory pathways that input to *COOLAIR* expression that lead to the induction of *COOLAIR* in response to prolonged cold.

5.4.5 Bursts of *COOLAIR* expression in response to repeated sub-zero spikes may reduce *FLC* levels

Unspliced and spliced *FLC* levels did not respond in the short term to sub-zero temperatures however spliced *FLC* was reduced much more by the fluctuating regime that included exposure to sub – zero temperatures. Bursts of *COOLAIR* transcription in response to these sub – zero temperatures could be influencing the expression of *FLC* resulting in a reduction in spliced *FLC* expression over time.

5.5 Future Experiments

5.5.1 Temperature profiles to dissect how *COOLAIR* measures two weeks of average 5°C

COOLAIR is induced in the long term to an average daily temperature of 5°C according to the temperature profiles tested in this Chapter. It is not clear from this experiment what *COOLAIR* may be responding to. Further experiments investigating *COOLAIR* induction under different temperature regimes may allow elucidation of the specific thermosensory inputs required for *COOLAIR* induction in response to prolonged cold.

COOLAIR may be responding to low temperatures experienced during the night time. To test this *COOLAIR* expression could be measured over a day after two weeks vernalisation at constant 5°C, fluctuating 5°C (3°C) (as used in the experiments above) and in response to a fluctuating 5°C (3°C) regime where the higher temperatures occur during the night and the lower temperatures occur during the day (Figure 5-6A). Although this regime is likely unrealistic in nature, in a lab setting it could reveal when *COOLAIR* is responsive to cold temperatures.

Alternatively, *COOLAIR* may respond to an absence of warm temperatures, similar to one of the thermosensory inputs at VIN3 (Antoniou-Kourounioti et al., 2018). In the regimes tested day time temperature never went above 12°C and *COOLAIR* expression was similar between the regimes tested. A spike of 20°C could prevent *COOLAIR* expression remaining high despite low temperature experienced throughout the rest of the day (Figure 5-6B). This spike could be tested during the day and night and the influence it has on *COOLAIR* expression recorded.

Finally, *COOLAIR* could be responding to the 'number of hours per day' exposed to cold temperature. In another experiment, *COOLAIR* expression could be measured in response to constant 5°C and then in regimes where the plants spend different amounts of time at 5°C (Figure 5-6C).

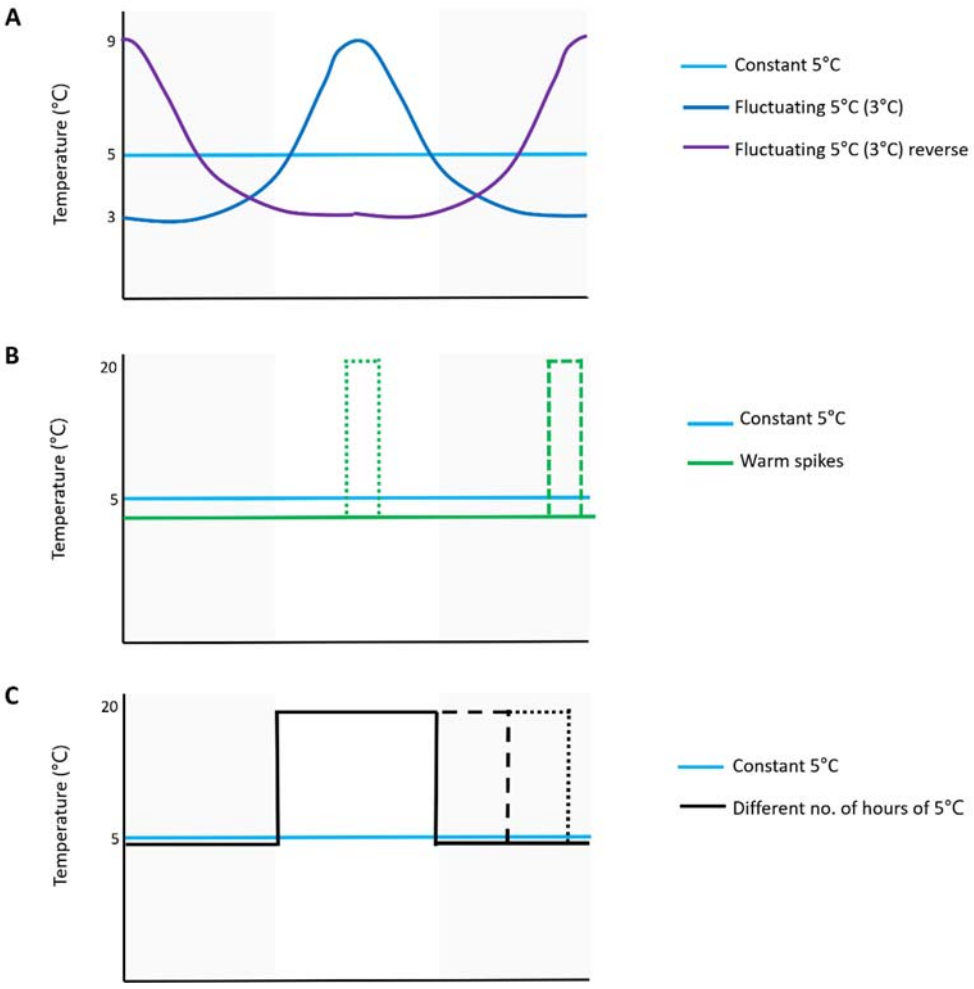


Figure 5-6 Potential temperature profiles to dissect thermosensory inputs at *COOLAIR*
Diagrams representing the temperature profiles across 24-hour (8-hour light, 16-hour dark) days of different vernalisation conditions to test *COOLAIR* expression after two weeks. A) Fluctuating 5°C (3°C) regimes that can investigate the role of night time temperatures in inducing *COOLAIR*. B) Investigating whether warm temperature spikes repress *COOLAIR* expression the average daily temperature still being 5°C. C) Exposing plants to different lengths of constant 5°C.

6 Discussion

My project aimed to investigate how *COOLAIR* is induced in response to cold temperature during vernalisation in *Arabidopsis thaliana*. *COOLAIR* induction is important in the transcriptional shutdown of *FLC*, which allows the efficient nucleation of epigenetic silencing marks. These epigenetic marks silence the *FLC* locus so that after vernalisation *FLC* is no longer expressed, FT repression is lifted, and the plant is permitted to flower.

Specifically, this thesis had two primary aims: to identify and characterise candidate factors required for *COOLAIR* induction and to dissect the temperature inputs detected by pathways to influence *COOLAIR* expression. Prior to this work, *COOLAIR* was known to be induced by two weeks of constant 5°C by an unknown mechanism. My work identified multiple candidate factors involved in *COOLAIR* induction by utilising forward and reverse genetic approaches (Chapter 3) and has further characterised the potential mechanism of two of these candidates, the CBFs and ARP6 (Chapter 4) by detailed analysis of *COOLAIR* expression over a vernalisation time course. Additionally, my work isolated at least two thermosensory inputs to the regulation of *COOLAIR* expression, namely massive induction in response to short – term exposure to sub-zero temperatures and maintained induction over weeks of cold with an average daily temperature of 5°C (Chapter 5).

The results presented in this thesis will be summarised below and framed in the context of the aims of this thesis first described in the introduction. Implications of this research, relevant to future *COOLAIR* research and research within the wider field, will also be detailed below.

6.1 Identification of regulators of cold-induced *COOLAIR* expression

The set of lncRNAs antisense to *FLC*, *COOLAIR*, have been known to be induced by the early weeks of vernalisation for over a decade yet the mechanism of their induction has remained elusive (Swiezewski et al., 2009). In fact, a thermosensor for the process of vernalisation has not yet been identified.

To identify candidate thermosensors, a forward genetic mutant screen, based on the induction of a translatable *COOLAIR*: LUC reporter fusion, was undertaken. Interestingly few mutants that had impaired induction of *COOLAIR*: LUC could be recovered. Those that were recovered all expressed *COOLAIR*: LUC more highly before cold and induced *COOLAIR*: LUC less in response to cold (2273,

6722 and 2265) implicating a mechanism whereby *COOLAIR* repressors are themselves inactivated by vernalisation conditions causing *COOLAIR* induction.

An alternative approach investigating the binding of trans factors to the *COOLAIR* promotor identified the possible role of redundant families of transcription factors being important for *COOLAIR* induction. The redundancy of these factors could explain why few mutants impaired in *COOLAIR*: LUC induction were identified from the screen.

Ultimately, the two approaches led to the identification of multiple candidate regulators of cold – specific *COOLAIR*: LUC induction. These included transcription factors (CBFs, CAMTAs and NTL8) and histone remodelling complex proteins (ARP6) and some as yet unidentified factors (6722).

CBFs are well known master regulators of the cold acclimation pathway but few studies have investigated the role of CBFs in prolonged cold exposure. CBFs will be described in more depth in section 6.2. CAMTAs have been implicated in the induction of SA biosynthesis genes in response to weeks of cold temperature. This parallels the induction of *COOLAIR* observed after weeks of cold exposure. Additionally, CAMTA3 activates expression of CBF1 and CBF2. The observation that both CBFs and CAMTAs may bind the COOLAIR promotor could represent a novel feedback system between CBF and CAMTA proteins when controlling expression of the same target.

NTL8 is a very interesting *COOLAIR* regulator to follow up given that it also plays a role in VIN3 induction in response to vernalisation conditions. NTL8, perhaps redundantly with NTL14 and NTL5, could represent a vernalisation sensitive regulator that is important for both *COOLAIR* and VIN3 induction, directly linking transcriptional shutdown and nucleation of epigenetic silencing marks at *FLC*.

ARP6 was a somewhat surprising discovery from the mutant screen as it is already known to be required for high *FLC* expression. ARP6 is part of the SWR1 complex that deposits H2A.Z into chromatin. H2A.Z is present at both the 5’ and 3’ end the *FLC* locus and it was thought that H2A.Z was important for *FLC* expression. This thesis has shown that in fact H2A.Z may be equally important controlling *COOLAIR* expression, repressing it until exposure to vernalisation conditions. H2A.Z dynamics have not been investigated in response to cold temperature despite broadly controlling gene expression in response to warm temperature. The potential role of ARP6 in *COOLAIR* induction could reveals the need for this to be investigated.

Overall then the identification of multiple candidate factors presented in this thesis could launch the investigation of the mechanism by which all these factors may impact *COOLAIR* expression. In the wider field, temperature sensing for the cold acclimation pathway and the vernalisation pathway have been tentatively linked whilst further study into H2A.Z dynamics in response to low temperature could reveal further insights into the role of H2A.Z and thermosensing.

6.2 Dissection of *COOLAIR* induction in the identified mutants

In Chapter 4, candidate *COOLAIR* regulators ARP6 and the CBFs were investigated more closely by tracking expression of *COOLAIR*: LUC and endogenous *COOLAIR* over multiple time points during vernalisation.

This showed that *COOLAIR* is not induced by one or subsequent weeks of cold in a *cbfs* triple mutant. This implicates the CBF factors in activating *COOLAIR* expression in response to cold. *CBF* expression was also shown to increase from NV to one, two and three weeks of cold, revealing that the CBFs may not just play roles in gene expression changes that happen in response to less than 24 hours exposure to cold. This reveals the need for further work into the expression levels and roles of CBF factors in prolonged cold periods. The cold acclimation pathway was claimed to be independent from vernalisation however the evidence in this thesis would support a role for the CBFs in inducing *COOLAIR*, presumably affecting the transcriptional shutdown of *FLC*, one stage of vernalisation (Bond, Dennis, & Finnegan, 2011).

The *arp6* (2265) mutation is epistatic to cold temperature on *COOLAIR*: LUC expression highlighting a role for ARP6 or H2A.Z in repressing *COOLAIR* during NV conditions but permitting *COOLAIR* expression in response to vernalisation conditions. ARP6 has not been implicated in controlling *COOLAIR* expression before though it has been associated with controlling *FLC* expression for over a decade. This is probably because at the endogenous locus, in *arp6,* the effect on *COOLAIR* is subtle and could be easily overlooked. This is likely due to the complex feedback associated with the *FLC/ COOLAIR* locus. Again, using a reporter *COOLAIR* gene that is independent from *FLC* has revealed the primary direct effects of a mutation on *COOLAIR* expression. Future work should confirm the localisation of H2A.Z at *COOLAIR*: LUC and endogenous *COOLAIR* to reveal H2A.Z dynamics in response to vernalisation conditions. It may also be interesting for the wider field to investigate the redistribution of H2A.Z genome wide in response to cold temperature.

6.3 Investigation of *COOLAIR* induction in response to different temperature regimes

COOLAIR induction had only been investigated in response to vernalisation lab conditions that are highly unrealistic relative to field conditions. A large – scale field experiment provided insights into how vernalisation progresses outside of highly controlled conditions and revealed surprisingly that *COOLAIR* is induced by sub-zero temperatures.

This thesis successfully showed that *COOLAIR* is induced in response to short-term sub – zero temperatures rapidly, transiently and repeatedly. It is not clear from current research how this could influence *FLC* expression however this thesis did reveal that in response to repeated exposure to sub – zero temperatures, spliced *FLC* was reduced much more than in a fluctuating regime without sub – zero spikes. This thesis did not uncover a mechanism for how *COOLAIR* is induced by freezing temperatures though this appears to be an independent thermosensor from that of the induction of *COOLAIR* in response to prolonged cold. It may involve changes in chromatin dynamics given the phenotype observed in *arp6*. The CBFs are not implicated in this induction.

The fluctuating regimes used to test *COOLAIR* induction also revealed that induction of *COOLAIR* in response to prolonged cold likely responds to an average daily temperature of 5°C. In the future various other temperature regimes can be used in an attempt to isolate the true components of temperature being sensed to cause *COOLAIR* induction.

Coupled with work concerning temperature sensing at VIN3 (Antoniou-Kourounioti et al., 2018), this thesis supports the existence of distributive temperature sensing for induction of regulators during vernalisation. The hunt for a single thermosensor for vernalisation is probably not realistic. In environments where temperature is constantly fluctuating and differing from year to year it may be more reliable to use multiple sensors to interpret the temperature profile that feeds into a process as important as vernalisation.

6.4 Conclusion

The silencing of *FLC* in response to vernalisation is a powerful paradigm for epigenetic control of gene expression in response to an environmental stimulus. *COOLAIR* induction has emerged as an

important component of the transcriptional shutdown phase of *FLC* silencing. Yet the temperature inputs and thermosensors responsible for this induction have not be uncovered.

This thesis has described identification of multiple candidate regulators for *COOLAIR* induction and further characterised the role of CBF transcription factors and the SWR1 histone remodelling complex in *COOLAIR* induction. Additionally, at least two different temperature inputs to *COOLAIR* induction have been identified. This thesis has also described work showing how thermosensing at *COOLAIR* is likely distributive with *COOLAIR* induction occurring as a result of multiple converging pathways.

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8 Appendix

qPCR primers

Luciferase	F - TAACGATCAGTTCGCCGATG
	R and RT - AGCCATTGTTTGGATCGT TT
Total COOLAIR	F - ACGTCCCTGTTGCAAAATAAGC
	R and RT - TGCATCGAGATCTTGAGTGTATGT
Proximal COOLAIR	RT - TGGTTGTTATTTGGTGGTGTG
	F - TCACACGAATAAGGTGGCTAATTAAG
	R - CCTGCTGGACAAATCTCCGA
Spliced FLC	F - AGCCAAGAAGACCGAACTCA
	R and RT - TTTGTCCAGCAGGTGACATC
Unspliced FLC	F - CGCAATTTTCATAGCCCTTG
	RT and R - CTTTGTAATCAAAGGTGGAGAGC
CBF1	F - AGTCAACATGCGCCAAGGAT
	RT and R - ATGTCCAGGCCATGATTCG
CBF2	F - TGACGTGTCCTTATGGAGCTA
	RT and R - CTGCACTCAAAAACATTTGCA
CBF3	F - CGCTGACTCGGCTTGGA
	RT and R - GCATCACACATCTCATCCTGAAAC
UBC	F - CTGCGACTCAGGGAATCTTCTAA
	RT and R - TTGTGCCATTGAATTGAACCC
PP2A	F - ACTGCATCTAAAGACAGAGTTCC
	RT and R - CCAAGCATGGCCGTATCATGT

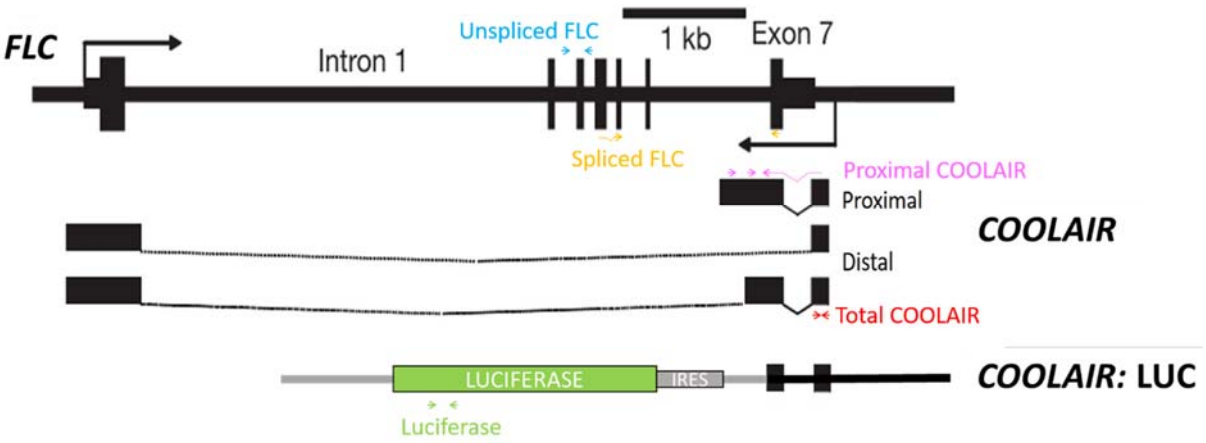

Figure 8-1 – qPCR primers at the *FLC/COOLAIR* locus and *COOLAIR:LUC* – Forward and reverse primer location for qPCR expression analysis of total *COOLAIR* (red), proximal *COOLAIR* (pink), Spliced *FLC* (yellow), unspliced *FLC* (blue) and luciferase (green). RT primers used are the reverse in all cases except for proximal *COOLAIR* – additional RT primer shown.