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Title: The role of *FLOWERING LOCUS C* in vernalization of *Brassica*: The importance of vernalization research in the face of climate change

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Running head: Vernalization in *Brassica*

Abstract

As climatic changes occur over the coming decades, our scientific understanding of plant responses to environmental cues will become an increasingly important consideration in the breeding of agricultural crops. This review provides a summary of the literature to date regarding vernalization research in *Brassicaceae*, covering both the historical origins of vernalization research and the current understanding of the molecular mechanisms behind the regulatory pathways involved in vernalization and subsequent inflorescence. In addition, we discuss the evolutionarily conserved biology between the model organism *Arabidopsis thaliana* and the *Brassica* genus of crop cultivars and contrast the differences between the genera to illustrate the importance of *Brassica* specific research into vernalization.

Additional keywords: Vernalization, Flowering time, *FLOWERING LOCUS C*, Histone modification, Epigenetics

Introduction

As sessile organisms, plants are reliant primarily upon environmental cues. Various species of angiosperms (flowering plants) have, over the course of evolutionary history, evolved molecular mechanisms that maximize their reproductive success (Yan *et al.* 2003; Groover 2005). The proper timing of the transition from vegetative to reproductive growth is key to ensuring the successful propagation of offspring. For example, some species of plants require cross-pollination for successful reproduction due to self-incompatibility (Fujimoto and Nishio 2007; Kim *et al.* 2009). Therefore, their flowering must occur synchronously among the individuals of the species. Flowering should also occur at a time when there are pollinators present to carry out such cross-pollination activity. Depending upon a plant's native climatic environment, flowering must take place when the local weather conditions allow the relatively delicate floral structures to develop and persist, followed by environmental conditions

that will permit seeds to reach maturity (Huijser and Schmid 2011).

Several environmental cues play decisive roles in determining the developmental fate of plants. In temperate climates, many winter-annual, biennial, and perennial plants utilize cold exposure as an environmental cue to synchronize seasonal flowering. Numerous plant species require prolonged cold exposure, generally encountered during the course of a winter season, prior to flowering and setting seed. Without exposure to a prolonged cold period, flowering is blocked. This process, known as vernalization, was first mentioned in the scientific literature in 1918 by the German plant physiologist Gustav Gassner in a paper on the effect of lowered temperature on the development of winter rye (Gassner 1918). A decade thereafter, while working at the Azerbaijan agricultural research station, the Russian agronomist Trofim Lysenko published his 1928 work on the effect of cold exposure on flowering in wheat. The term jarovization, derived from the Russian term jarove meaning "Spring cereals", was used to describe the process now referred to as vernalization (Amasino 2004). In the English language, the term vernalization, a translation of the term jarovization, is derived from the Latin word *vernalis*, meaning "of, relating to, or occurring in the Spring" (Chouard 1960). The vernalization requirement is an evolutionary adaptation to temperate climates, preventing flowering prior to encountering a winter season and ensuring flowering under the more favorable weather conditions of the spring season. Vernalization requiring plants often also possess a long day (LD) photoperiod requirement. The coupling of a vernalization requirement to a LD photo-period requirement ensures that the decreasing day lengths of the autumn season places a further inhibition against precocious flowering, favoring flowering as the day-length increases during spring (Amasino and Michaels 2010).

Within the Linnaean taxonomic classification system, the *Brassicaceae* family of angiosperms comprises a set of scientifically and agriculturally important crop cultivars. *Brassicaceae* is a moderately-sized taxonomic family, with 338 genera and 3,709 accepted plant species (Warwick *et al.* 2006), and the model plant, *Arabidopsis*

thaliana, resides within the *Brassicaceae* family. The genomes of three members of the genus Brassica, *Brassica rapa*, *Brassica nigra*, and *Brassica oleracea,* are denoted as the A, B, and C genomes, respectively. The agriculturally important allotetraploid *Brassica napus* is derived from the interspecific hybridization of the A and C genomes of *B. rapa* and *B. oleracea*, respectively. With the advent of genomic sequencing, the genetic relationship between the three diploid species, *B. rapa*, *B. nigra*, and *B. oleracea*, in the Brassica genus has been elucidated further, revealing they are descended from a common hexaploid ancestor that underwent a whole genome triplication (WGT) event roughly 15.9 million years ago (MYA), with speciation divergence occurring approximately 4.6 MYA (Cheng *et al.* 2014; Liu *et al.* 2014).

Plant species within the *Brassicaceae* family, specifically *B. rapa* and *B. oleracea*, exhibit either a biennial or a perennial reproductive cycle, with a long day facultative photoperiod requirement coupled to a vernalization requirement. *B. rapa* crop cultivars include Chinese cabbage, komatsuna, ~~bok choy,~~ pak choi, mizuna, and turnip. *B. oleracea* contains the crop cultivars cauliflower, broccoli, cabbages, Brussels sprouts, kohlrabi, and kales. The regulation of flowering time is important for stable production in agricultural foods. For example, leafy vegetables such as Chinese cabbage or cabbage benefit from an increased vernalization requirement as a prolonged vegetative growth state allows for an increase in leaves and reductions in early bolting: both of which are desired agricultural traits. In this review, we describe the recent research findings on vernalization in Brassica, especially focusing on crops such as Chinese cabbage (*B. rapa)* and cabbage (*B. oleracea)* with a late bolting requirement.

The molecular basis for vernalization in *A. thaliana*

Regulation of flowering time is well studied in the annual model species, *A. thaliana*. Within the complex regulatory networks that comprise the floral pathway, the central repressor of the transition from the vegetative state to inflorescence is FLOWERING LOCUS C (FLC) in *A. thaliana*. *FLC* encodes a MADS box DNA-

binding protein that acts as a floral repressor (Michaels and Amasino 1999; Sheldon *et al.* 1999), and its expression is regulated by various genes involved in several flowering pathways (Figure 1). *Brassicaceae* includes many perennial species such as *Arabis alpina* and *Arabidopsis halleri*, and the respective *FLC* orthologs are also key regulators of flowering transition with seasonal gene expression (Wang *et al.* 2009; Aikawa *et al.* 2010).

The FRIGIDA pathway and the autonomous pathway

Two antagonistic autonomous pathways regulate the pre-vernalization levels of *FLC* expression in *A. thaliana* (Crevillén and Dean 2011). *FLC* is negatively regulated by a group of genes collectively known as the autonomous pathway (Sung and Amasino 2005). In *A. thaliana*, the autonomous pathway is composed of the following proteins, FCA, FPA, FY, FVE, LUMINIDEPENDENS (LD), FLOWERING LOCUS D (FLD), and FLOWERING LATE KH DOMAIN (FLK) (Figure 1) (Kim *et al.* 2009; Crevillén and Dean 2011). In contrast to other regulatory pathways composed of a hierarchical set of activities, the autonomous pathway is composed of parallel pathways of genes with differing biochemical functions. However, these proteins all share *FLC* as a target (Figure 1). For example, one such parallel pathway involves the flowering time control proteins FCA and FY, which interact to regulate RNA processing of *FLC* (Marquardt *et al.* 2006; Liu *et al.* 2007).

In 1950, the annual winter habit of *A. thaliana* was mapped predominantly as a monogenic trait to the *FRIGIDA (FRI)* locus (Härer 1950). There are natural variations of the vernalization requirement, and *FRI* is one of the causative genes of this variation. Rapid cycling accessions have mutations in *FRI*, and loss-of-function mutations have originated independently (Johanson *et al*. 2000; Méndez-Vigo *et al.* 2011). While laboratory accessions like Columbia and Landsberg *erecta* carry non-functional *FRI* alleles (*fri*), the addition of an active *FRI* allele markedly upregulates *FLC* expression and re-establishes a vernalization requirement (Kim *et al.* 2009). FRI

acts as a scaffold protein interacting with FRIGIDA LIKE 1 (FRL1), FRIGIDA ESSENTIAL 1 (FES1), SUPPRESSOR OF FRIGIDA 4 (SUF4), and FLC EXPRESSOR (FLX) that assembles a large protein complex, FRIGIDA-containing complex (FRI-C). SUF4 can directly bind to the *FLC* promoter, and FRI-C activates *FLC* expression (Figure 1) (Choi *et al.* 2011). The current understanding of the molecular actions by which *FRI* upregulates *FLC* is via a co-transcriptional mechanism that interacts directly with the nuclear cap-binding complex (CBC), thereby increasing the proportion of 5' methyl-capped messenger RNA (mRNA) *FLC* transcripts. This increased capping activity provides subsequent increases to both *FLC* transcript stability within the nucleus, and enables increased nuclear export of *FLC* transcripts for translation by the ribosomes located at the rough endoplasmic reticulum (RER) (Geraldo *et al.* 2009). In winter annuals, the *FRI* pathway acts epistatically to the autonomous pathway via the up-regulation of *FLC* expression to create vernalization-responsive late flowering (Koornneef *et al.* 1991). In rapid-cycling accessions, which typically lack functional *FRI* alleles, the autonomous pathway represses *FLC*. Repression of *FLC* results in a loss of the vernalization requirement. However, recessive autonomous pathway mutants, due to high levels of *FLC* expression, result in a late flowering phenotype and possess a vernalization requirement (Feng *et al.* 2011). Therefore, the regulatory function of both the FRI and the autonomous pathways acting together upon FLC serve to set the basal levels of *FLC* expression via constitutive activation and repression, respectively.

FLC as the molecular basis for vernalization

FLC forms protein complexes with SHORT VEGETATIVE PHASE (SVP), and possibly other currently unknown proteins, to repress the expression of the floral pathway integrators, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *FLOWERING LOCUS T (FT)* genes, that initiate flowering in *A. thaliana* (Figure 1) (Helliwell *et al.* 2006; Mateos *et al.* 2015). *FLC* expression is regulated by several

genes and is reduced by vernalization. Several mutants, which affect the acceleration of flowering by vernalization, have been identified. *VERNALIZATION 1* (*VRN1*) and *VRN2* are required for the maintenance of *FLC* repression by vernalization, but their expression levels are not changed by vernalization (Kim *et al.* 2009). In contrast, *VERNALIZATION INSENSITIVE 3 (VIN3)* expression is induced by vernalization, and *VIN3* acts as the initial transcriptional repressor of *FLC* after cold exposure (Figure 1). However, because of the transient expression of *VIN3* in response to cold, it cannot maintain this repression (Sung and Amasino 2004; Kim *et al.* 2009).

Prior to vernalization, active histone marks such as histone H3 and H4 acetylation (H3ac and H4ac, respectively) and the trimethylation of the lysine amino acids at positions four and thirty-six (H3K4me3 and H3K36me3, respectively) are present at the *FLC* locus (Bastow *et al.* 2004; Sung and Amasino 2004; Yang *et al.* 2014). SET-domain proteins such as ARABIDOPSIS TRITHORAX 1 (ATX1), ATX2, ATX-related 7 (ATXR7), and EARLY FLOWERING IN SHORT DAYS (EFS), which are involved in trithorax group (trxG) proteins, catalyze H3K4me3 and H3K36me3 at the *FLC* locus (Figure 1) (Kim *et al.* 2009; Tamada *et al.* 2009; Buzas *et al.* 2012). FRI-C recruits general transcriptional factors and chromatin modifiers including ATX1, EFS, and the SWR1 complex, which is involved in the replacement of H2A with an activity-associated H2AZ variant, suggesting that FRI-C leads to the active histone marks (H3K4me3, H3K36me3, H3ac, and H4ac) in the *FLC* gene resulting in activation of *FLC* expression (Figure 1) (Noh and Amasino 2003; Deal *et al*. 2007; Jiang *et al*. 2009; Choi *et al.* 2011). Mutations in the *EARLY FLOWERING 7* (*ELF7*) and *ELF8*, which are components of the RNA polymerase II-Associated Factor 1 (PAF1)-containing complex, cause a reduction of *FLC* expression and H3K4me3 (Figure 1). Consequently, the PAF1 complex acts in the maintenance of active histone modifications at the *FLC* locus (He *et al.* 2004).

Exposure to prolonged cold, for four or more weeks, initiates a series of transcriptional changes within the plant, leading to the epigenetic silencing of *FLC*

(Song *et al.* 2012). This silencing is mediated by Polycomb repressive complex 2 (PRC2) containing VRN2, which acts to enrich H3K27me3 at the *FLC* locus (Jiang *et al.* 2008; Young *et al.* 2011; Coustham *et al.* 2012). These markers form the histone coding template that, in turn, initiates chromatin re-modeling complexes, which convert the *FLC* genomic locus from a euchromatic state (H3K4me3 and H3K36me3) to heterochromatic state (H3K27me3) (Simon and Kingston 2009). This functionally silences the *FLC* gene, preventing further transcription and subsequent translation of the FLC protein (Schmitz and Amasino 2007; Müller and Goodrich 2011). TERMINAL FLOWER 2/LIKE HETEROCHROMATIN PROTEIN 1 (TFL2/LHP1) is specifically associated with H3K27me3 (Turck et al. 2007), and the *lhp1* mutant disrupts the maintenance of *FLC* silencing by vernalization, suggesting that *LHP1/TFL2* might be a component of the epigenetic silencing machinery at *FLC* (Figure 1) (Mylne *et al.* 2006)

During exposure to cold, several regulatory transcriptional processes take place in a specific order, leading to PRC2-mediated gene silencing (Figure 1). Upon initial cold exposure ~~induction~~, the anti-sense *cis*-regulatory long non-coding RNA (lncRNA) *COOLAIR* is transcribed, concomitantly, an initial down-regulation of *FLC* transcription occurs (Swiezewski *et al.* 2009). *COOLAIR* is considered to be involved in the autonomous pathway and the PRC2-mediated epigenetic silencing of *FLC*, but its function in the cold-induced silencing of *FLC* is still controversial (Liu *et al.* 2010; Angel *et al.* 2011; Helliwell *et al.* 2011). After the degradation of *FLC* mRNA, *COOLAIR* transcription is then reduced by the formation of a RNA-DNA hybrid within its promoter, R-loop (Sun *et al.* 2013). After reduction of *COOLAIR* expression, the sense *cis*-regulatory lncRNA *COLDAIR*, whose transcriptional start site is located at intron 1 of the *FLC* locus, is upregulated. This, in turn, is believed to recruit PRC2 proteins to the *FLC* locus, in order to begin PRC2-mediated silencing and the chromatin remodeling activity that functionally silences *FLC* transcription (Heo and Sung 2011; He 2012; Ietswaart *et al.* 2012; Kim and Sung 2012; Csorba *et al.* 2014). *FLC* haplotypes in *A. thaliana*, as defined by variation of the non-coding sequence in intron 1,

have been shown to be linked to their ecotypes (Li *et al.* 2014). This is most likely due to the selective pressure of geographic climatic variation with respect to the length and severity of a region's winter season.

Similar but different: the molecular basis of vernalization in *Brassica*

Of particular interest is the natural variation of vernalization requirements found amongst members of the *Brassicaceae* family. Experimental evidence shows that the molecular activities of flowering pathways, as elucidated in *A. thaliana*, are largely conserved in *Brassic* (Irwin *et al.* 2012; Xiao *et al.* 2013; Fadina and Khavkin 2014). The *FLC* gene is highly conserved among members of the *Brassicaceae* family and serves to maintain a plant's vegetative growth until exposure to prolonged cold has occurred (Zou *et al.* 2012).

QTL studies into the early- and late-flowering phenotypes of *Brassica* cultivars revealed that several loci co-localized with the orthologs of well-known flowering genes found in *A. thaliana*; including multiple orthologs of *FLC*, *FRI*, and *FT* that contribute to the flowering time trait in *Brassica* (Axelsson *et al.* 2001; Lou *et al*. 2007; Okazaki *et al.* 2007; Wang *et al.* 2009; Wang *et al.* 2011; Kakizaki *et al.* 2011). Comparison of loci found in *A. thaliana* to QTLs from each of the diploid *Brassica* species (*B. rapa*, *B. nigra*, and *B. oleracea*) with those found in amphidiploid species (*B. juncea* and *B. napus*), through the alignment of map positions, allowed for cross-species analysis of genes within the QTL regions (Osborn *et al.* 1997; Axelsson *et al.* 2001).

In the reference genome of *B. rapa* (A genome), Chiifu-401-42, four *FLC* paralogs, Bra006051 (*BrFLC3*), Bra009055 (*BrFLC1*), Bra022771 (*BrFLC5*), and Bra028599 (*BrFLC2*), were found (Figure 2, 3). However Bra022771 is possibly non-functional because of deleted exons (Figure 3) (Wang *et al.* 2011). Two reference genomes in *B. oleracea* (C genome) were reported with two *BoFLC* (Bol008758, Bol043693) paralogs found in *B. oleracea* var. *capitata* homozygous line 02-12 and four *BoFLC* paralogs found in TO1000DH3, a doubled haploid derived from a rapid

cycling *B. oleracea* (Figure 2) (Liu *et al.* 2014; Parkin *et al.* 2014). In the reference genome of *B. napus* (AC genome), nine *FLC* paralogs were found in the European winter oilseed cultivar Darmor-*bzh* with four *FLC*s in the A_n subgenome and five within the C_n subgenome (Figure 2) (Chalhoub *et al.* 2014).

Genetic mutation of Brassica FLC genes may cause flowering-time variation

The genetic redundancy of *Brassica FLC*s provides diversity of the vernalization response and flowering time through an accumulation of various mutations at these loci. Flowering properties are often linked to agriculturally important traits in *Brassica* vegetables with varieties possessing or lacking heading and curd formation. QTL analyses for various flowering properties and genetic sequence studies have indicated that the variation of flowering time may be explained by the sequence diversity among the *Brassica FLCs* (Li *et al*. 2009; Kakizaki *et al*. 2011; Kitamoto *et al*. 2014).

In *B. rapa*, co-localization of flowering-time QTLs and *BrFLC* genes have been reported in several populations (Osborn *et al*. 1997; Kole *et al*. 2001; Schranz *et al*. 2002; Lou *et al*. 2007; Li *et al*. 2009; Zhao *et al*. 2010; Kakizaki *et al*. 2011; Kitamoto *et al*. 2014). Using an F_2 population derived from a cross of an early flowering oilseed rape and a late flowering leafy vegetable, two major QTLs were detected close to *BrFLC1* and *BrFLC2* (Li *et al*. 2009). In the early flowering parent, the abundance of *BrFLC2* transcript was reduced. This was most likely due to nucleotide substitution(s) occurring upstream of the start codon. Furthermore an alternatively spliced *BrFLC1* transcript was detected (Li *et al*. 2009). This alternative splicing of *BrFLC1* has also been observed in other early flowering accessions, and a SNP at the 5' splice site of the sixth intron was also commonly detected (Yuan *et al*. 2009; Li *et al*. 2009). In an F_2 population derived from the cross of an early bolting parent and an extremely late bolting parent, QTLs for bolting time after vernalization co-localized with the late bolting alleles of *BrFLC2* and *BrFLC3*. These two genes, both of which carry large

insertions in the first intron, have been shown to exhibit less sensitive silencing dynamics in response to vernalization (Kitamoto *et al*. 2014). Several reports have highlighted the contribution of *BrFLC2* in determining flowering time, especially in oil type *B. rapa* (var. *oleifera* and var. *tricolaris*) (Lou *et al*. 2007; Zhao *et al*. 2010; Wu *et al*. 2012; Xiao *et al.* 2013). In addition, there is a report of QTL analysis showing that *BrFLC1* and *BrFLC5* are also considered to be important (Kakizaki *et al*. 2011). Therefore, these findings lead to the conclusion that the candidate *FLC* paralog(s) found during QTL analyses may be dependent upon the combination of parental lines because whichever mutation(s) exist at different *FLC* loci could affect the total functional *BrFLC* dosage, resulting in a divergent flowering time, given that all *BrFLC* paralogs function as floral repressors as partly shown by Kim *et al*. (2007).

In *B. oleracea*, *BoFLC2* was located via a QTL analysis of flowering time in F_2 progeny derived from a cross of broccoli (*B. oleracea* var. *italica*), with one parent having a frame-shift caused by a single base deletion in exon 4 of the gene. This might affect the functionality of gene product in the early flowering parent (Okazaki *et al*. 2007). In F_2 progeny derived from a cross of cauliflower (*B. oleracea* var. *botrytis*) with differences in curd development and flowering time, the genotype of *BoFLC2,* with or without the same mutation, explained 40% of the flowering-time variance (Ridge *et al.* 2014). Moreover, QTL and subsequent functional and genomic sequence analyses in purple sprouting broccoli have shown that nucleotide polymorphisms in *BoFLC2* are responsible for altering the vernalization response, especially after returning plants to ambient temperature (Irwin *et al.* 2016). These reports revealed the central role of *BoFLC2* in determining vernalization response and flowering time in *B. oleracea* (Okazaki *et al*. 2007; Ridge *et al*. 2014; Irwin *et al*. 2016). However, it is possible that polymorphisms at the remaining *BoFLC* paralogs (*BoFLC1, BoFLC 3,* and/or *BoFLC 5*) also contribute to flowering time variation.

In seed crops such as rapeseed/canola (*B. napus*), the production of seed depends on flowering time, thus the adaptation of flowering time is important for

breeding. There is natural variation in flowering time in canola, and most of it is due to response to vernalization; three groups, spring type, winter type, and semi-winter type, were distinguished by their vernalization response (Raman *et al.* 2016). Four *FRI* genes were identified in *B. napus*, and *BnaA.FRI.a* was co-localized with a major flowering time QTL (Wang *et al.* 2011). Transformation of *AtFLC* in early flowering *B. napus* results in late flowering, and constitutive expression of *BnFLC1-5* in *A. thaliana* delayed flowering (Tadege *et al.* 2001). These results indicate the BnFRIs and BnFLCs have a similar function of AtFRI and AtFLC, respectively. More recently, genome wide association studies (GWAS) into the flowering times of *B. napus* has further reinforced some of these comparative genomics results. Several SNPs associated with flowering time were detected within 20 kb regions of *FT*, *FLC*, and *FRI* using 188 accession of *B. napus* collected from different geographic location around the world (Raman *et al.* 2016). However in contrast to these findings, a GWAS using 158 European winter-type *B. napus* inbred lines revealed that *FLC* was absent from the candidate regions associated with flowering time (Schiessl *et al*. 2015). Other known flowering time regulators found in *A. thaliana* were found to contribute to the flowering time phenotype (Schiessl *et al*. 2015; Raman *et al.* 2016), providing further evidence for a largely conserved regulatory gene network between the *Arabidopsis* and *Brassica* genera.

FLC remains a key molecular component of vernalization in Brassica

All four *BrFLC* paralogs were expressed in leaves grown under normal conditions in *B. rapa*; three of the *BrFLC* paralogs (*BrFLC2*, *BrFLC3*, and *BrFLC5*) had H3K4me3, while *BrFLC1* had both H3K4me3 and H3K36me3. After vernalization, expression of all four *BrFLC* genes was reduced, and the repression of the *BrFLCs* was maintained following a return to ambient temperatures (Figure 4). Accumulation of H3K27me3 was observed in *BrFLC1*, *BrFLC2*, and *BrFLC3*, and H3K27me3 levels were stably maintained after returning to warm conditions (Kawanabe *et al.* 2016). This

indicates that as in *A. thaliana*, in *B. rapa* the silencing of *BrFLC* paralogs is associated with increased H3K27me3 and that the repression of *BrFLCs* is maintained by H3K27me3.

The nucleotide sequences of various *FLC* paralogs in *B. rapa*, *B. oleracea*, and *B. napus* are highly conserved (Figure 2) (Zou *et al.* 2012). While the alignment of upstream sequences with more than 75% sequence identity showed divergence in the promoter regions and length of intronic regions (Figure 3) (Zou *et al.* 2012). While variations within the promoter regions appear to affect the transcriptional levels of *FLC* paralogs in *B. rapa* (Hong *et al.* 2011), *FLC* transcriptional levels of three of the paralogs (*BrFLC1*, *BrFLC3*, and *BrFLC5*) do not appear to determine if a particular variety is early- or late-flowering (Franks *et al.* 2015). By contrast, there is a difference in the gene expression levels among *FLC* paralogs in *B. rapa* (Kim *et al.* 2007; Kawanabe *et al.* 2016). There are polymorphisms in *BoFLC2* that alter the sensitivity and silencing dynamics of *BoFLC2* expression in response to the length of cold exposure, suggesting that polymorphisms in *BoFLC2* are responsible for heading time variations in *B. oleracea* (Irwin *et al.* 2016). In *A. thaliana*, the first intron in addition to the promoter region and exon 1 is important for the maintenance of *FLC* repression (Sheldon et al., 2002), and lncRNA *COLDAIR* is expressed from the mid-region of the first intron (Tsai *et al*. 2010; Heo and Sung 2011). Natural variation of vernalization response is due to the noncoding *cis* variation within *FLC* in *A. thaliana* (Coustham *et al*. 2012; Li *et al*. 2015). Sequence variations within the first intron appears to delay bolting in *B. rapa* (Kitamoto *et al.* 2014), but the intron 1 region of the *FLC* paralogs in *B. rapa* and *B. oleracea* lack homology to the *A. thaliana COLDAIR* sequence.

Similar to *A. thaliana*, over-expression of *FLC* natural antisense transcripts (NATs) results in a decreased flowering time and decreased levels of *FLC* expression, suggesting that *BrFLC* NATs suppress the activity of the *BrFLC2* gene in response to cold (Li *et al.* 2016). Unlike *A. thaliana*, *Brassica* has been shown to utilize a different set of NATs. *Brassica* transcribes three class I (convergent) NATs (*BrFLC2as406*,

BrFLC2as599, *BrFLC2as477*) and two class II (convergent) NATs (*BrFLC2as816* and *BrFLC2as755*), whose names are based on their respective transcript lengths. Interestingly, the transcriptional start site (TSS) of these sequences is located 169 nucleotides downstream of the 3-prime end of the *BrFLC2* gene (Figure 4). Compared to the TSS of the *COOLAIR* sequence in *A. thaliana*, these NATs were further away from the transcriptional stop sites of their respective *FLC* gene. Furthermore, the NATs identified in *B. rapa* appear to only be transcribed at the *BrFLC2* locus (Li *et al.* 2016). Further study will be required to show whether the induction of *BrFLC2* NATs are directly involved in the suppression of *BrFLC2*.

Currently, the complete regulatory roles of these NATs in the epigenetic silencing of *FLC* in *Brassica* have yet to be elucidated, but the experimental evidence to date suggests a more complex functional role than that of *A. thaliana*.

Perspective

The projected future climatic shifts, brought about by anthropogenic climate change, will pose many challenges to agriculture over the coming decades (Hatfield *et al.* 2014). Agricultural and biological research will therefore play an increasingly important role in the adequate adaptive responses required to meet these challenges. While the general implications of increased levels of atmospheric carbon are well understood, the precise effects of global climate change with respect to modern agriculture are still poorly understood. However, the effects on agricultural climatic zones as environmental conditions change, mean breeders will need to adapt and respond to increased environmental stresses placed upon crop cultivars. Fomenting cooperation between climate scientists, biologists, and breeders through cross-disciplinary research into the impacts of climatic changes on key agricultural cultivars will better prepare us for the challenges we may expect to face in the upcoming decades, as well as serve to enrich the current understanding of fundamental plant biological functions.

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Figure legends

Figure 1. Diagram illustrating regulation of *FLOWERING LOCUS C* (*FLC*) involves a complex network of pathways in *A. thaliana*. FLC acts as the central integrator of signaling that acts to represses flowering.

Figure 2. Phylogenetic tree of *FLC* genes in *Brassica* by maximum likelihood method (Kimura 1980). The percentage of trees in which the associated taxa clustered together is shown next to the branches (Kumar *et al*. 2016).

Figure 3. Diagram illustrating the gene structure for the reported *FLC* genes in *A. thaliana* (TAIR10), *B. rapa* (Chiifu-401-42), *B. oleracea* (*B. oleracea* var. *capitata* homozygous line 02-12), and *B. napus* (Darmor-*bzh*). Exons are represented by black boxes and solid lines represent introns. The nucleotide lengths of the exons

and introns are drawn to a scale in this image.

Figure 4. The comparison of molecular dissections for vernalization response between *A. thaliana* and *B. rapa*. a. Schematic illustrations of transcripts from the *AtFLC* and the *BrFLC2* loci. The black boxes (coding regions) and the white boxes (untranslated regions) represent exons, and the solid lines indicate introns. The dashed line represents sense lncRNA, *COLDAIR*. Gray boxes indicate alternatively spliced antisense lncRNA, collectively named *COOLAIR* in *A. thaliana* and *BrFLC2as* in *B. rapa*. b. The gene expression dynamics in response to vernalization. The solid lines, the chain lines, and the dashed line represent the relative expression levels of *AtFLC/BrFLCs*, antisense transcripts (*COOLAIR/BrFLC2as*), and *COLDAIR*, respectively. The dotted lines indicate the upregulation of *AtVIN3*, encoding a plant homeodomain protein, and its orthologous *BrVIN3* during cold exposure.