Chapter 6

Genetic and Epigenetic Regulation of Vernalization in Brassicaceae

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Additional information is available at the end of the chapter

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Abstract

A wide variation of morphological traits exists in *Brassica rapa* L. and *Brassica oleracea* L., and cultivated vegetable varieties of these species are consumed worldwide. Flowering time is an important agronomic trait in these species and varies among varieties or cultivars. Especially, leafy vegetable species need a high bolting resistance. Isolation of *FLOWERING LOCUS C* (*FLC*), one of the key genes involved in vernalization, has now provided an insight into the molecular mechanism involved in the regulation of flowering time, including the role of histone modification. In the model plant *Arabidopsis thaliana*, *FLC* plays an important role in modulating flowering time. The response to vernalization causes an increase in histone H3 lysine 27 tri-methylation (H3K27me3) that leads to reduced expression of the *FLC* gene. *B. rapa* and *B. oleracea* both contain several paralogs of *FLC* at syntenic regions identified as major flowering time and vernalization response quantitative trait loci (QTL). We introduce the recent research, not only in *A. thaliana*, but also in the genus *Brassica* from a genetic and epigenetic view point.

Keywords: vernalization, flowering time, FLOWERING LOCUS C, histone modification, high bolting resistance

1. Introduction

During the life cycle of plants, the change from vegetative to reproductive growth is a major developmental transition in angiosperms. Flowering is the process where a transformation of the vegetative stem primordia into floral primordia occurs due to biochemical changes. In

most plants, once the transition from vegetative to reproductive growth begins, it cannot be reversed. Thus, the proper timing of this transition is advantageous to ensure the successful propagation of offspring. Internal (endogenous cues) and external (environmental stimuli) factors both play important roles in flowering time. As plants are sessile organisms, plants are greatly affected by environmental conditions such as day length (photoperiodism) and temperature. Photoperiodism is controlled via the photoreceptor proteins phytochrome and/or cryptochrome, responsible for sensing red/far-red and blue light, respectively [1]. We typically refer to photoperiod requirements as either long day (LD) or short day (SD) with respect to the length of time that a plant receives daylight. As this photoperiod signal is also tied to the annual cyclical seasonal changes, LD, coinciding with the spring and summer seasons, and SD, associated with the autumn and winter seasons, both play roles in the floral development of several plant species [2]. The regulation of flowering to changes in temperature is known as vernalization. Vernalization is the process that accelerates flowering in response to the prolonged cold winter. Many plants have a vernalization requirement and will actively repress flowering until after an exposure to prolonged cold. This acts to synchronize seed production with the favorable environmental conditions of spring. The presence of certain photoperiods and ambient temperatures after vernalization are also important [3, 4].

Brassica is a genus in the family of Brassicaceae and includes 37 species of flowering plants. Many of these are important both economically and as agricultural crops, with members such as broccoli, brown mustard, brussels sprouts, cabbage, cauliflower, Chinese cabbage, kale, kohlrabi, rape, rutabaga, and turnip. The crops from this genus are sometimes known as cole crops. Three members of the genus *Brassica*: *Brassica rapa*, *Brassica nigra*, and *Brassica oleracea* are denoted as the A, B, and C genomes, respectively. These three species share a unique genomic relationship known as the "Triangle of U" [5]. Allotetraploids between these three species contain two complete diploid genomes derived from the two different parental species, one diploid genome from each parent. The agriculturally important allotetraploid *Brassica napus* (canola or rapeseed) 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 three diploid species such as *B. rapa*, *B. nigra*, and *B. oleracea*, in the *Brassica* genus has been elucidated further, revealing that they are descended from a common hexaploid ancestor that underwent a whole genome triplication event roughly 15.9 million years ago (MYA), with speciation divergence occurring approximately 4.6 MYA [6].

Different cultivated varieties of the diploid species of *B. rapa* exhibit extreme developmental and morphological diversity, and from the organs consumed they are generally divided into leafy, turnip, and oil types. *B. rapa* crops are normally grown in two seasons, autumn and spring, and their flowering habits are generally controlled by day length and/or temperature. *B. rapa* is a facultative LD plant. Although LD photoperiod conditions accelerate its flowering, it can also flower under SD photoperiod conditions [7]. *B. rapa* is a leafy vegetable, and flowering time is an important developmental trait because bolting can occur before plants reach the harvest stage. Examples include Chinese cabbage and pak choi, where early bolting markedly impairs the product value. Early bolting mostly occurs due to low temperatures at the beginning of cultivation and the longer day lengths during the growing period of the spring

season. Thus, the genetic dissection of flowering time control is central to the breeding of late bolting leafy *B. rapa* cultivars. *B. oleracea* (cabbage), a plant-vernalization-responsive species, has become established as one of the most valuable vegetable crops in the Brassicaceae family and is widely consumed by both humans and livestock [8, 9]. Vernalization can be classified into two types: seed-vernalization-responsive and plant-vernalization-responsive, according to the age at which the plant vernalizes in response to low temperature [9]. In the plant-vernalization-responsive type, biennial plants grow vegetatively in the first year and flower in the following year after winter. The vernalization of cabbage normally requires low temperatures of approximately 6–8 weeks in duration that is initiated at the stage of seven to nine leaves, or when the stem diameter reaches 6 mm for the initiation of flowering [8, 9]. The differences in the mechanisms involved in vernalization and flowering between seed- and plant-vernalization-responsive types is of agronomic and scientific interest to understand. As such, attempts have been made to transfer the seed-vernalization character from Chinese cabbage (*B. rapa*) into cabbage (*B. oleracea*) [10], and the plant-vernalization character from cabbage into Chinese cabbage [11]. *B. napus* is an important oilseed crop in the temperate regions of the world. The production of seed in canola depends upon flowering time, thus the adaptation of flowering time is important for breeding. In *B. napus*, the natural variation in flowering time in response to vernalization was characterized into three groups: spring type, winter type, and semi-winter type [12].

Understanding the molecular mechanism(s) responsible for vernalization in the control of flowering is important for the breeding of high bolting resistance in *B. rapa* and *B. oleracea* leafy vegetables. Recent studies on vernalization using *Arabidopsis thaliana*, one of the model organisms used for studying plant biology and the first plant to have its entire genome sequenced, provided key insight into the molecular mechanism of vernalization. The knowledge derived from *A. thaliana* research has been useful for understanding the molecular mechanism of vernalization in the genus *Brassica*. In this chapter, we describe the latest research findings on vernalization in *A. thaliana* and the *Brassica* genus, especially leafy vegetables such as Chinese cabbage (*B. rapa*) and cabbage (*B. oleracea*) with a high bolting resistance.

2. Vernalization research in model plant *Arabidopsis thaliana*

A. thaliana is a small dicotyledonous species used as a model organism for studying plant biology belonging to the family Brassicaceae. In *A. thaliana*, over 180 genes are implicated in flowering time control and these genes are categorized into six major pathways that control flowering time, including the photoperiod/circadian clock pathway, vernalization pathway, ambient temperature pathway, age pathway, autonomous pathway, and gibberellin pathway [13, 14]. It is a much-studied model for vernalization and the transition to the reproductive phase of *A. thaliana* occurs by two related events, the floral transition (initiation of the first flower) and the bolting transition (elongation of the first internode) [15]. Brassicaceae includes many perennial species such as *Arabis alpina* and *Arabidopsis halleri*, and the respective *A. thaliana* orthologous gene is key regulator of flowering transition with seasonal gene expression [16, 17]. In this section, we introduce research on vernalization in *A. thaliana*.

2.1. Genes involved in vernalization

Two key genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), have been identified by map-based cloning of naturally occurring early flowering accessions of *A. thaliana*. Rapid cycling accessions have mutations in *FRI*, and loss-of-function mutations have originated independently [3, 18]. The functional *FRI* gene acts upstream of the *FLC* expression within the vernalization pathway. 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). These proteins assemble to form a large protein complex, FRIGIDA-containing complex (FRI-C), with SUF4 directly binding to the *FLC* promoter and FRI-C activating *FLC* expression [19].

The *FLC* gene is a floral repressor that contains a MADS box transcriptional regulator protein domain, and maintains a plant's vegetative growth until exposure to prolonged cold is experienced. Within the vegetative apical meristem, FLC interacts with several important genes during vegetative growth by inhibiting the activation of a set of genes required for the transition of the apical meristem to inflorescence, ultimately determining the plant's reproductive fate [20–22]. At the molecular level, FLC blocks flowering by binding to genes that promote flowering and repressing their transcription. Initially, three flowering time genes, *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSSON OF CONSTANS 1* (*SOC1*), and *FLOWERING LOCUS D* (*FD*) were reported to be targeted by FLC, with FLC binding to the promoters of *SOC1* and *FD* and to the first intron of *FT* [23, 24]. Later, using antiserum raised against the FLC protein without the conserved MADS domain, more putative *FLC* targeted genes were identified at the whole genome level by chromatin immunoprecipitation sequencing (ChIP-seq). About 500 *FLC* binding sites, mostly located in the promoter region of genes containing one CArG box (the known target of MADS-box proteins) were identified [25]. Two genes (*FT* and *SOC1*) that function downstream of the flowering activator CONSTANS (CO) in the photoperiod pathway were identified as being negatively regulated by *FLC* [4, 14].

In addition to the previously mentioned *FRI*-dependent pathway, the autonomous pathway is also known to repress *FLC* expression. In the autonomous pathway, key genes such as *FCA*, *FLD*, *FLOWERING LATE KH DOMAIN* (*FLK*), *FPA*, *FVE*, *FY*, and *LUMINIDEPENDENS* (*LD*) were identified [4, 22, 26], and mutation in these genes results in the activation of *FLC* and a late flowering phenotype. Thus, *FRI* and the autonomous pathway are internal regulators of basal *FLC* expression via constitutive activation and repression, respectively.

Examination of the regulation of *FLC* by the vernalization pathway in various vernalization-responsive accessions and flowering time mutants of *A. thaliana* showed that the levels of *FLC* mRNA and protein correlated well with flowering time in response to cold treatment [3, 4, 22]. To identify the genes involved in the vernalization pathway, mutants that do not respond to vernalization were characterized. Two mutants termed *vernalization 1* (*vrn1*) and *vrn2* were identified [3, 27, 28]. Cold treatment reduced the *FLC* expression levels in *vrn1 fca-1* or *vrn2 fca-1* double mutants. However, when plants are returned to a warm environment, the suppression of *FLC* expression was not maintained. This suggests that VRN1 and VRN2

are involved in the maintenance of *FLC* repression by vernalization, but not in the initial repression [27, 28]. *VRN1* encodes a nuclear protein with B3 domains, a highly conserved plant-specific transcription factor that binds to DNA [28]. *VRN2* encodes a nuclear-localized zinc-finger protein showing a similarity to Polycomb Group (PcG) proteins of plants and animals [27]. The expression levels of *VRN1* and *VRN2* are not changed by vernalization. However, a third gene involved in the repression of *FLC* by vernalization, *VERNALIZATION INSENSITIVE 3* (*VIN3*), is activated by vernalization, and *VIN3* encodes a plant homeodomain (PHD) finger containing protein [29].

2.2. Epigenetic regulation of *FLC* gene

Epigenetic regulation is defined as changes in gene activities that are inherited through cell divisions without alteration in the DNA sequence. Epigenetic regulation is crucial for the development and adaptation of plants to the changing environment [30, 31]. DNA methylation and histone modification are the best examples of epigenetic modifications. The fundamental subunit of chromatin is the nucleosome, and the nucleosome consists of 147 base pairs of DNA wrapped around an octamer of histone proteins comprised of two tetramers. Each of the two tetramers contains one of each of the core histone proteins H2A, H2B, H3, and H4. The alteration of chromatin structure, which causes changes in transcription, is regulated by various post-translational modifications such as methylation or acetylation of the N-terminal regions of the histone proteins [32]. Histone lysine residues can be mono-, di-, or tri-methylated, and each methylation state is associated with different functions [32]. In plants, histone deacetylation, H3K9me2, and H3K27me3 are associated with gene repression, and histone acetylation, H3K4me3, and H3K36me3 are associated with gene activation [22, 31, 33, 34].

The vernalization response is one example of epigenetic regulation, and *FLC* expression is regulated by chromatin modification [34, 35]. *FLC* is expressed before prolonged cold exposure, and H3K4me3 or H3K36me3 is associated with activation of *FLC* expression [36]. FRI-C facilitates recruitment of chromatin-modifying factors to *FLC*, such as the chromatin remodeling SWR1 complex (delivering H2A.Z variant) or the histone methyltransferases EARLY FLOWERING IN SHORT DAYS (EFS) (a homolog of SET2 that catalyzes H3K36me3) [19], ARABIDOPSIS TRITHORAX LIKE 1 (ATX1) (which catalyzes H3K4me3) [37], and ARABIDOPSIS TRITHORAX-RELATED 7 (ATXR7) [38]. H3K4me3 activity is also mediated by the yeast RNA polymerase II (Pol II) Associated Factor 1 (PAF1) complex, histone H3K4 methyltransferases such as ATX1, ATX2, and ATXR7, and the complex protein associated with Set 1 (COMPASS)-like complex that contains WDR5 HOMOLOG A (WDR5a), EFS, and ARABIDOPSIS Ash2 RELATIVE (ASH2R) [22, 39].

Prolonged cold exposure induces VIN3, a PHD-finger protein, which acts to establish the initial repression of *FLC* [29]. PHD-finger proteins VIN3, VRN5, VIN3/VRN5-like 1 (VEL1) interact with VRN2 protein and form POLYCOMB REPRESSIVE COMPLEX2 (PHD-PRC2) complex [29, 40, 41]. *FLC* repression by vernalization is associated with the enrichment of H3K27me3, which is mediated by the PHD-PRC2 mechanism [41]. During prolonged cold

exposure, H3K27me3 is enriched in chromatin at the *FLC* transcription/translation start sites [42]. After cold exposure, during growth at higher temperatures, the H3K27me3 modification extends across the *FLC* gene [42]. The initial transcriptional repression of *FLC* is PRC2-independent, but the stable maintenance of repression requires PRC2 [27]. The maintenance of *FLC* silencing under warm conditions after cold exposure is therefore mediated by PHD-PRC2 spreading H3K27me3 over the *FLC* locus. In addition, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), which is associated with H3K27me3, and VRN1 are required for the maintenance of stable *FLC* repression [28, 43, 44].

FLC is epigenetically silenced by vernalization, but *FLC* needs to be reactivated to restore the requirement for vernalization in each subsequent generation. *FLC* expression is repressed in gametogenesis, regardless of the parental state of vernalization, and its expression increases as the embryo develops [45, 46]. Some autonomous pathway genes, which upregulate *FLC* in vegetative tissues, are required for *FLC* expression in the early embryo [46]. In contrast, *FRI* and *SUF4* are not required to reset the expression of *FLC*, however, they are required to maintain it after reactivation of *FLC* [46]. EARLY FLOWERING 6 (ELF6) is a jumonji domain H3K27me3 demethylase protein and is expressed at high levels in both flowers and embryos, but at low levels in seedlings [47]. Resetting of the vernalized state at the *FLC* locus in the next generation requires H3K27 demethylation by ELF6 [47]. However, *FLC* expression in some non-vernalized *elf6* mutants was found to be lower than in non-vernalized wild type, but the expression level was fully restored in the next generation [48]. Thus, there may be another factor associated with the resetting of *FLC* expression. LEAFY COTYLEDON1 (LEC1) encodes a seed-specific NF-YB transcription factor that is a subunit of NF-Y that binds to NF-C and NF-A, and regulates embryogenesis. LEC1 NF-Y engages EFS, which is associated with H3K36me3, and the SWR1 complex, remodeling the chromatin state at the *FLC* locus to a transcriptionally active euchromatic state during embryogenesis [46, 48]. This activity suggests that LEC1 NF-Y binds to the *FLC* promoter, displacing Polycomb proteins and recruiting EFS, and that the maintenance of a euchromatic state at the *FLC* locus by LEC1 inhibits the transmission of repressive chromatin marks [48].

2.3. Long noncoding RNA induced by cold treatments in the *FLC* locus

Advanced technologies such as tiling arrays or RNA-sequencing (RNA-seq), use high-throughput sequencing to enable the discovery of long noncoding transcripts. It has been shown that some long noncoding RNAs (lncRNAs) are involved in the regulation of gene expression through interactions with associated proteins. Several PRC2-associated lncRNAs have been identified in mammals, for example, *XIST* targets PRC2 to the X chromosome or HOTAIR targets PRC2 to the *HOX* gene, resulting in silencing of target genes [22]. Using a custom array covering the 50 kb region around *FLC*, with single-nucleotide resolution of both strands, lncRNAs termed cold-induced long antisense intragenic RNAs (COOLAIR) have been identified. COOLAIR encompasses most of the *FLC* locus, from the 5′ start to the 3′ polyadenylation sites, and COOLAIR is alternatively polyadenylated and spliced. The induction of COOLAIR occurs after 14 days of cold treatment in wild type and *vin3–4* mutants,

which is earlier than *VIN3* induction (20 days after transfer to cold), and the suppression of unspliced sense *FLC* transcription was observed before the maximum induction of *VIN3* [29, 49]. COOLAIR promoter-driven antisense transcription of a reporter gene could lead to transient cold-induced repression, suggesting that COOLAIR contributes to the early repression of sense *FLC* transcription transiently before the stable repression that is mediated by the PHD-PRC2 complex [49]. However, plants having T-DNA insertions in the region covering COOLAIR where COOLAIR expression or upregulation of COOLAIR is not observed during cold treatment, showed normal repression of sense *FLC* by vernalization. This suggests that the production of COOLAIR transcripts is not an essential component of vernalization-induced repression of *FLC* [50]. Because of this, while COOLAIR is considered to be involved in the autonomous pathway and the PRC2-mediated epigenetic silencing of *FLC*, its function in the cold-induced silencing of *FLC* is still controversial [50–52]. After the degradation of *FLC* mRNA via COOLAIR, COOLAIR transcription is then reduced by the formation of an RNA-DNA hybrid within its promoter, R-loop [53].

Another lncRNA, COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR), has been identified in the first intron of *FLC* in the sense direction. COLDAIR contains a 5′ cap structure but is not polyadenylated. COLDAIR is induced during cold exposure and reaches its maximum level of expression at around 20 days of cold exposure. The expression level then returns to the pre-vernalized level after more than 30 days of cold. The induction of COLDAIR occurs earlier than *VIN3* induction, but later than the induction of COOLAIR. The transcription start site of COLDAIR is located within the VERNALIZATION RESPONSE ELEMENT (VRE), a region important for the stable repression of *FLC* by vernalization [44]. COLDAIR interacts with *FLC* chromatin and one of the components of the PRC2 complex, CURLY LEAF (CLF), specifically during cold exposure. Reduced COLDAIR by RNA interference showed that *FLC* repression was not maintained when plants were returned to a warm growth condition after vernalization. COLDAIR mutants decrease the association of PRC2 and H3K27me3 accumulation [54]. In addition, the repression of *FLC* expression by cold treatment was not maintained in the COLDAIR mutants once the plants were moved to normal growth conditions [55]. Increased expression of *CLF* and enrichment of H3K27me3 by vernalization were not observed in knockdown lines of COLDAIR, indicating that COLDAIR plays a role in the establishment of the stable maintenance of *FLC* repression during vernalization by recruitment of the PHD-PRC2 complex to *FLC* chromatin [54].

RNA immunoprecipitation (RIP) using the antibody against CLF followed by a tiled RT-PCR identified COLDWRAP (cold of winter-induced noncoding RNA from the promoter) overlapping the promoter region of *FLC*. COLDWRAP associated with PRC2 throughout cold exposure. COLDWRAP transcripts increased during cold exposure, and were maintained even after cold exposure. COLDWRAP has a 5′ cap structure but is not polyadenylated. COLDWRAP is 316 bp in length, and its transcription start site is 225 bp upstream from the *FLC* mRNA transcription start site. COLDWRAP mutants showed an absence of stable repression of *FLC* expression by cold exposure, with a low level of recruitment of PRC2 and H3K27me3 accumulation, suggesting that COLDWRAP is involved in PRC2-mediated *FLC* silencing by vernalization [56].

3. Vernalization research in the genus *Brassica*

Flowering time is very important for the harvest of valuable agricultural products because the flowering that is induced by exposure to cold temperatures, known as bolting, can occur. This is especially the case in vernalization-sensitive leafy vegetables of the genus *Brassica* (*B. rapa* and *B. oleracea*). Because bolting causes the devaluation of agricultural products, a high bolting resistance is of economic significance for cultivar breeding. Additionally, the control of flowering time is also critical for the yield of seeds in canola (*B. napus*) because the appropriate timing of flowering maximizes both seed production and quality. The *FLC* gene maintains a plant's vegetative growth phase until exposure to prolonged cold, and is highly conserved among members of the Brassicaceae family [57], suggesting that the *FLC* gene is an important factor for the breeding of highly bolting resistant cultivars in leafy vegetables. Indeed, previous studies have already supported this possibility. This section introduces the research of flowering in the genus *Brassica,* mainly focusing on the *FLC* gene.

3.1. Species in the genus *Brassica* has the paralogs of *FLC* genes

Recently, the whole genome sequences of the diploid species, *B. rapa*, *B. nigra*, and *B. oleracea*, and the allotetraploid species, *B. napus* and *B. juncea* have been determined. From these genome sequences, it is already known that there are multiple *FLC* paralogs in the genus *Brassica.* Four *FLC* paralogs, Bra009055 (*BrFLC1*), Bra028599 (*BrFLC2*), Bra006051 (*BrFLC3*), and Bra022771 (*BrFLC5*) were found in the reference genome of *B. rapa*, Chiifu-401-42, but Bra022771 is possibly a pseudogene because of the two deleted exons. Two *BoFLC* (Bol008758, Bol043693) paralogs are found in the *B. oleracea* var. *capitata* homozygous line 02–12, while four *BoFLC* paralogs are found in TO1000DH3, a doubled haploid derived from a rapid cycling *B. oleracea*. In the reference genome of allotetraploid species 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 [58].

3.2. QTL controlling flowering time

In the genus *Brassica*, several quantitative trait loci (QTLs) affecting flowering time have been identified. To identify the genes involved in flowering time QTLs, populations derived from parents that show differences for flowering time were used.

In *B. rapa*, several QTLs for flowering time (VFR1, 2, and 3 in non-vernalized condition and FR1, 2, and 3 in vernalized condition) were identified using an F_2 population derived from a cross between an annual and a biennial oil seed cultivars [59, 60]. Later, VFR2 and FR1 were located in the regions covering *BrFLC1* and *BrFLC2*, respectively [61, 62]. Using a multi-population derived from several parental lines (rapid cycling, Chinese cabbage, yellow sarson, pak-choi, and a Japanese vegetable turnip variety) eight QTLs for flowering were detected, and one major QTL co-localized with *BrFLC2* [63]. *BrFLC1* and *BrFLC2* were linked to QTLs that control bolting, budding, and flowering time using an F_2 population derived from an early flowering oilseed rape line, yellow sarson, and a late flowering line of the Japanese

commercial komatsuna variety, osome [64]. In yellow sarson, a decrease in *BrFLC2* transcripts was observed and was considered to be due to a nucleotide substitution occurring upstream of the start codon [64]. QTL analyses using other parental combinations between pak-choi and yellow sarson also showed the co-localization of a major QTL with *BrFLC2* [65, 66]. QTL analysis using a recombinant inbred line population produced from a cross between a caixin line (L58, ssp. *parachinensis*) and a yellow sarson line (R-o-18, ssp. *tricolaris*) detected two QTLs in both the spring and autumn seasons, and the *BrFT2* gene is co-localized with a QTL. Later flowering is caused by a transposon insertion in the second intron of *BrFT2*. In another QTL, *BrFLC2* was located, and the earlier flowering line has a 57-bp deletion covering the fourth exon and fifth intron [67]. QTL analyses by different groups over many years have shown that a major QTL of flowering time co-localized with *BrFLC2*. Because of the early flowering line, yellow sarson was used as a parent for making the populations for QTL analysis in all groups, it seems likely that all the groups detected the defects of *BrFLC2* function in yellow sarson as a flowering time QTL. QTL analysis using an F_2 population derived from a cross between an extremely late bolting line (Nou 6 gou, PL6) and early bolting line (A9709) of Chinese cabbage was performed in two different conditions, greenhouse and open field. Five QTLs were detected, but the QTLs did not map to the same position between the two conditions. Three of five QTLs were co-localized with *BrFTa* (greenhouse), *BrFLC1* (open field), and *BrFLC5* (open field) [68]. In another parental combination in Chinese cabbage, an F_2 population was developed from the cross of an early bolting parent of commercial F_1 varieties, Early, and an extremely late bolting breeding line, Tsukena No. 2. In this analysis, the QTLs for bolting time after vernalization co-localized with the late bolting alleles of *BrFLC2* and *BrFLC3*. These two genes carry large insertions in the first intron, suggesting that a weak repression of *BrFLC2* and *BrFLC3* transcripts by vernalization causes the extremely late bolting of Tsukena No. 2 [69]. Furthermore, this group succeeded in developing new F_1 hybrids of Chinese cabbage by introducing these two *FLC* alleles from Tsukena No. 2 [70].

In *B. oleracea*, QTL analysis using a population derived from a DH line of broccoli, Green Comet (var. *italica*), and a DH line of cabbage, Reiho (var. *capitata*), identified a major QTL covering *BoFLC2*, while *BoFLC1, BoFLC3,* and *BoFLC5* were not linked to the QTLs [71]. In addition, Green Comet (non-vernalization type) has a single base deletion in exon 4 leading to the frame-shift, suggesting that *BoFLC2* contributes to the control of flowering time [71]. Another group performed QTL analysis using the population derived from a rapid cycling line of *B. oleracea* var. *alboglabra* (A12DHd) and the broccoli variety, Green Duke. Because these two lines contain non-functional copies of *BoFLC2* (named *BoFLC4* in this paper), there is a deletion in the A12DHs, and a single base deletion in exon 4 in Green Duke, it was concluded that *BoFLC2* is not responsible for the flowering time difference between the two lines [72]. Later, the association between flowering time (under both glasshouse and field conditions) and a QTL at *BoFLC2* has been shown using the population derived from two purple sprouting broccoli lines, E5 and E9; E9 requires longer cold periods than E5 to head [73].

QTL analysis was also performed in *B. napus*, and QTLs for flowering time were co-localized with the genes involved in flowering time in *A. thaliana*. Using a population derived from a biennial rapeseed cultivar, Major, and the annual canola cultivar, Stellar, four QTLs (VFN1, 2, 3 in non-vernalized condition and FN1 in vernalized condition) were detected. One major

QTL, VFN1, co-localized with the region collinear with the top of chromosome 4 in *A. thaliana* covering the *FRI* gene [60, 74]. Six *FT* paralogs have been mapped in the *B. napus* genome and three (*BnA2.FT, BnC6.FT.a*, and *BnC6.FT.b*) genes were co-localized with two major QTL clusters for flowering time using populations from the European winter cultivar, Tapidor, and the Chinese semi-winter cultivar, Ningyou 7 [75]. Using the same population, *BnaA.FRI.a* was co-localized to a major flowering time QTL in multiple environments [76]. QTL analyses were performed under field and greenhouse conditions using a population from two Australian *B. napus* cultivars, Skipton and Ag-Spectrum, and the number of QTL detected differed between the two growth conditions. Flowering time genes such as *FLC* were localized within marker intervals associated with flowering time [77, 78]. A total of 158 European winter type *B. napus* inbred lines were genotyped to investigate the association with flowering time, plant height, and seed yield by a genome-wide association study (GWAS). This study revealed that the flowering time regulators, *Bna.FLC* and *Bna.CO*, were absent from the candidate regions associated with flowering time [79]. Another GWAS study examined the flowering times and genome architectures of 188 accessions of *B. napus* collected from different geographic locations around the world, showing associations between flowering time and regions within 20 kb of *FT*, *FLC*, and *FRI* [12].

3.3. Regulation of *FLC*s by vernalization in the genus *Brassica*

From QTL analyses, it has been demonstrated that multiple *FRI* or *FLC* paralogs are involved in the flowering times of *B. rapa*, *B. oleracea*, and *B. napus*. The transformation of *BoFRIa* complemented the loss of FRI function in *A. thaliana*, indicating that BoFRI has the same function as AtFRI [80]. In the case of *FLC* paralogs, the early flowering line yellow sarson has a non-functional *BrFLC2* [64, 67], and a naturally occurring splicing mutation in the *BrFLC1* gene is associated with flowering time variation [81]. In *B. oleracea*, an early flowering line of broccoli has a frame-shift mutation in exon 4 of *BoFLC2* [71]. In addition, 40% of flowering time variation in cauliflower (var. *botrytis*) was explained by the same mutation in *BoFLC2* [82]. Furthermore, transgenic plants overexpressing *BnFLC* paralogs in *A. thaliana* showed a late flowering phenotype, indicating that all five *BnFLCs* have similar function to *AtFLC* [83], and three *FLC*s (*BrFLC1*, *BrFLC 2*, and *BrFLC3*) have been confirmed to be a floral repressor in *B. rapa* [84]. These results indicate that FLC paralogs function as a floral repressor, and play an important role in the vernalization requirement.

In *B. rapa,* it has been shown that there is a difference in the expression levels of the *FLC* paralogs [85]. The coding sequences for the *FLC* paralogs are relatively conserved between *Brassica* species, but the alignment of the upstream sequences or introns are more divergent [57]. This suggests that these differences may account for the different steady state expression levels among *BrFLC* paralogs, or variation of the vernalization requirement.

In *B. rapa* grown under normal conditions, all four *BrFLC* paralogs were expressed in the leaves. The expression of *BrFLC* genes was reduced after vernalization, and the repression was stably maintained after returning to ambient temperatures. Before cold treatment, only *BrFLC1* showed accumulation of both H3K4me3 and H3K36me3 modifications, while three of the *BrFLC* paralogs (*BrFLC2*, *BrFLC3*, and *BrFLC5*) had only H3K4me3. After 4 weeks of cold

treatment, the accumulation of H3K27me3 was observed in *BrFLC1*, *BrFLC2*, and *BrFLC3*, and H3K27me3 was maintained after returning to a warm temperature [85]. These results indicate that, like *A. thaliana*, the repression of *BrFLC* expression by prolonged cold treatment was associated with the states of histone modification. The first intron, the promoter region, and exon 1 are important for *FLC* repression in *A. thaliana* [86], and lncRNA COLDAIR is expressed from the mid-region of the first intron in *A. thaliana* [54, 87]. Although insertions in the first intron cause a weak repression of *BrFLC2* and *BrFLC3* transcripts by vernalization in *B. rapa*, sequence similarity to the VRE in the first intron or to the COLDAIR of *A. thaliana* were not detected in the first intron of any of the *B. rapa* paralogs [69]. At least, COLDAIR-like transcripts in *B. rapa* were not detected. By contrast, COOLAIR-like transcripts were detected only from *BrFLC2*, and these transcripts were induced by cold treatment. The plant growth cycle was shortened by the over-expression of *FLC* natural antisense transcripts (NATs) (COOLAIR-like) resulting in decreased flowering time and *FLC* expression, suggesting that the activity of the *BrFLC2* gene was suppressed by *BrFLC* NATs during cold condition [88]. *BoFLC2* was shown to be a major determinant of heading date variation and vernalization response through allelic variation, and sequence polymorphisms in *BoFLC2* alter the sensitivity and silencing dynamics of its expression [73].

3.4. Perspective of vernalization research in the genus *Brassica*

In leafy vegetables such as Chinese cabbage or cabbage, a high bolting resistance is an important trait for cultivation, indicating that understanding the molecular mechanisms of the vernalization requirement is important for breeding. While research into vernalization and flowering time has provided a wealth of information, a complete understanding of the molecular mechanism controlling the vernalization requirement has not yet been elucidated. In contrast to *A. thaliana*, where histone modifications such as active marks, H3K4me3 and H3K36me3, or repressive marks, H3K9me2 and H3K27me3, have been characterized at the whole genome level by ChIP-seq, such analysis has yet to be conducted in the genus *Brassica*. Comparison of the histone modification states, especially H3K27me3, at the whole genome level between vernalized and non-vernalized plants will identify the genes other than *FLC*s involved in the regulation of vernalization. In addition, combining histone modification data with transcriptome data may facilitate the identification of genes involved in the regulation of vernalization. In *A. thaliana*, it has been revealed that lncRNAs such as COOLAIR, COLDAIR, and COLDWRAP are involved in vernalization [49, 54, 56]. Currently, COOLAIR-like transcripts were detected only from *BrFLC2*, and these transcripts were involved in the suppression of *BrFLC2* and maybe other *BrFLCs* [88]. However, in *B. rapa,* there is no report about the transcripts of COLDAIR or COLDWRAP, and regions sharing sequence similarity to the COLDAIR found in *A. thaliana* were not detected in the first intron of any of the *B. rapa* paralogs [69]. Therefore, there is a possibility that lncRNAs that do not show sequence similarity to COLDAIR or COLDWRAP may be involved in the regulation of repression of *FLC* in the genus *Brassica*. To examine this possibility, lncRNAs whose expression changes in response to vernalization will need to be assessed by RNA-seq. Thus, there exists a need to identify the sequences important for vernalization, termed VREs, within the genus *Brassica*; and to examine any sequence polymorphisms that may

exist with respect to the vernalization response. This will help to identify important regions and explicate their relationship to sensitivity of vernalization. If there are any correlations, they will be useful for marker-assisted selection, and serve as important tools for breeding in the genus *Brassica*.

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Conflict of interest

The authors declare that they have no conflict of interest.

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