Journal of Plant Biology
https://doi.org/10.1007/s12374-024-09430-y

RESEARCH ARTICLE

Time Course Transcriptomic Analysis of Cabbage (*Brassica oleracea* ssp. *capitata* L.) During Vernalization

Heewon Moon[1] · Byoung-Ho Hwang[2] · Minkyu Park[3] · Enamul Huq[4] · Dong-Hwan Kim[1]

Received: 6 February 2024 / Revised: 15 April 2024 / Accepted: 11 May 2024

Abstract

Long-term exposure to cold during the winter season, so-called vernalization, triggers the transition from the vegetative to the reproductive stage in many biennial and perennial plants. In the last decades, intensive researches have revealed the molecular mechanisms underlying this phenomenon, particularly using *Arabidopsis* model plant. Most Brassicaceae family plants, including the *Arabidopsis*, require vernalization for floral transition. Brassicaceae family plants can be classified into two groups: seed vernalization responsive type and plant vernalization responsive type. Cabbage belongs to plant vernalization responsive type. Molecular details on plant vernalization responsive trait of cabbage on vernalization are still poorly understood. In this study, we conducted a transcriptomic analysis of the cabbage inbred line ‘BN2348’ in response to vernalization. Similar to the case of *Arabidopsis*, two *VIN3* homologs (*BoVIN3.C3* and *BoVIN3.C2*) were highly induced by the exposure to long-term cold in *B. oleracea*. Our transcriptome analysis identified that two *FT* homologs (*BoFT.C2* and *BoFT.C6*) and three *SOC1* homologs (*BoSOC1.1.C4*, *BoSOC1.2.C4*, and *BoSOC1.C3*) were functioning for the regulation of floral transition in *B. oleracea*. In addition, by phylogenic and syntenic analyses, a total of five *FLC* homologs, named *BoFLC1.a*, *BoFLC1.b*, *BoFLC2*, *BoFLC3*, and *BoFLC5*, were identified in the genome of *B. oleracea*. Transcriptomic analysis indicated that these genes could be grouped into vernalization-responsive (*BoFLC2* and *BoFLC3*) and vernalization-insensitive genes (*BoFLC1.a*, *BoFLC1.b*, *and BoFLC5*). As green plant vernalization type, it might suggest the existence of vernalization-insensitive *BoFLC* homologs in young seedlings might be the reason why cabbage exhibits longer exposure of cold compared to seed vernalization type plants such as Chinese cabbage and *Arabidopsis*. These findings improve our understanding of the molecular dynamics underlying floral transition in cabbage plants.

Keywords Vernalization · RNA-seq · Cabbage · *Brassica oleracea* L. · Flowering time

Introduction

Brassicaceae family plants include many commercially valuable vegetable crops such as Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), Bok choi (*B. rapa* ssp. *chinensis*), turnip (*B. rapa* ssp. *rapa*), broccoli (*B. oleracea* L. var *italica*), cauliflower (*B. oleracea* L. var. *botrytis*), and cabbage (*B. oleracea* var. *capitata*) (Okazaki et al. 2007; Ridge et al. 2015; Irwin et al. 2016). Most Brassicaceae family plants can flower after a long-term exposure to cold, as in the winter season, called vernalization. However, their responses to cold vary depending on several developmental or environmental factors, including the developmental stage, temperature, and vernalization duration. Premature floral transition can induce severe damage in vegetable crops, reducing their commercial value by altering the nutrient content, texture, and taste. Hence, understanding the molecular mechanisms underlying floral transition is crucial for the development of new crop cultivars with valuable traits.

Vernalization can be grouped into two types depending on the plant stage that senses low temperature (Lin et al. 2005). One is called as the seed-vernalization-responsive type, in which plants can sense low temperatures at seed

✉ Dong-Hwan Kim
dhkim92@cau.ac.kr

[1] Department of Plant Science and Technology, Chung-Ang University, Anseong, Republic of Korea

[2] R&D Center, Asia Seed Co. LTD., Icheon-si 17414, Republic of Korea

[3] EuchroGene LLC, Clarksburg, MD 20871, USA

[4] Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, USA

Published online: 29 May 2024

germination and early seedling stage; the other is the plant-vernalization-responsive type, in which plants need to reach a certain growth stage before they become sensitive to low temperatures. In other words, plants belonging to plant-vernalization-responsive type cannot sense low temperature at seed germination and early seedling stage. Biennial plants that grow in a vegetative phase in the first year and flower in the next spring season after winter usually belong to the plant-vernalization-responsive type. Some species in the Brassicaceae family, such as *Arabidopsis* and *B. rapa* fall into the seed-responsive type, but several varieties in *B. oleracea* (i.e. cabbage and broccoli) belong to plant-vernalization-responsive type.

A study of QTL analysis identified *BoFLC2* (also referred to as *BoFLC4*) as a major gene for the vernalization of broccoli (Lin et al. 2005; Ridge et al. 2015). When the entire genomic sequence of *BoFLC2* from the promoter to 3′ downstream region was introduced into an *Arabidopsis FLC* null mutant, *flc-2* in FRI background transgenic lines completely complemented the rapid flowering behavior in winter-annual *Arabidopsis* plant (Irwin et al. 2016). Including BoFLC2, BoFLC1 and BoFLC3 were also shown to inhibit floral transition using *Arabidopsis* heterologous expression system (Lin et al. 2005). Another recent study has found that an *FLC* homolog, named *BoFLC1.C9* locus, which has a 67-bp DNA insertion in the second intron, is responsible for the late flowering phenotype in cabbage (*B. oleracea* var. *capitata*) cultivars (Abuyusuf et al. 2019). Other studies performing quantitative trait locus (QTL) analysis showed that *BoFLC1*, *BoFLC3*, and *BoFLC5* co-localize with major QTLs (Razi et al. 2008). Recently, it has been reported that *BoFLC3* contributes to the variation in curd formation in subtropical broccoli (*B. oleracea* var. *italica*) (Lin et al. 2018). Most recently, QTL-seq analysis revealed that tandemly duplicated *BoFLC1* which were located on chromosome 09 were responsible for the non-flowering phenotype of cabbage mutant called 'nfc' (Kinoshita et al. 2023). These observations indicated that BoFLC1 ~ BoFLC3 function as floral inhibitors and might confer the vernalization requirement in *B. oleracea* plants.

A group of floral integrator genes including *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) plays a positive role in the acceleration of floral transition in *Arabidopsis* (Yoo et al. 2005). *FT* and *SOC1* were identified as being suppressed by *FLC* (Lee and Lee 2010; Moon et al. 2005). It was demonstrated that FLC directly binds to the CArG box motif (CC(A/T)$_6$GG) located in the promoter sequence of the *FT* and *SOC1* promoter, which results in the suppression of *FT* and *SOC1* expression (Hepworth et al. 2002). In cabbage, two *FT* loci (named *BoFT.C2* and *BoFT.C6*) and three SOC1 homologs (*BoSOC1.C3*, *BoSOC1.1.C4*, and *BoSOC1.2.C4*) were identified and exhibit an increased level of expression after the vernalization treatment, in a manner similar to *Arabidopsis FT* and *SOC1* (Lin et al. 2005; Ridge et al. 2015; Irwin et al. 2016; Abuyusuf et al. 2019).

Wang et al. (2023) performed transcriptome analysis using two sets of cabbage materials that the early-flowering inbred line C491(P1) and late-flowering inbred line B602 (P2), the early-flowering individuals F2-B and late-flowering individuals F2-NB from the F2 population. As a result, the 9508 differentially expressed genes (DEGs) are revealed about both of C491_VS_B602 and F2-B_VS_F2-NB. Among them, Bo1g157450 (BoSEP2-1) and Bo5g152700 (BoSEP2-2) encoding MADS-box proteins, which function as key regulators in the repression of flowering, have been designated candidates for flowering timing regulation. Through qRT-PCR analysis, They showed significantly reduced expression in the late-flowering parent compared wite the early-flowering parent, that was consistent with the RNA-seq data. In addition, nucleotide sequence analysis of BoSEP2 genes revealed that three and one SNPs between late-flowering parent and early-flowering parent.

Even though the *BoFLC* clade genes are well conserved and demonstrated to be involved in flowering time control in *B. oleracea*, it is still poorly understood how these genes contribute to cabbage being the plant-vernalization responsive type. In this study, we performed time course transcriptomic analysis in response to vernalization. Our results provided a clue for the understanding on why cabbage (*Brassica oleracea* var. *capitata* L.) is classified into plant vernalization type, not seed vernalization type, requiring longer time for plant growth period prior to exposure to the vernalization to be competent for floral transition.

Results

Plant-vernalization Type Plant, Cabbage Exhibited a Different Vernalization Response with Seed-vernalization Type Plant like Chinese Cabbage and *Arabidopsis*

Aforementioned, Brassicaceae family plants can be classified into two groups based on the vernalization responsiveness, seed-vernalization responsive type and plant-vernalization responsive type. While *Arabidopsis* and Chinese cabbage fall into seed vernalization type, cabbage belongs to the plant vernalization responsive type. Generally, seed vernalization responsive type can successfully sense and remember the long-term cold at seed and early seedling stage. However, plant-vernalization type plants cannot memorize the exposure to long-term cold at the seed and early seedling stage, requiring certain level of vegetative maturation. To confirm this, we first measured flowering behavior of these plants after vernalization treatment. As expected, four leaf seedling of *Arabidopsis* and Chinese cabbage which were exposed to cold (4 ℃) for 40 days exhibited the rapid transition from vegetative stage to reproductive stage (Fig. 1A). Meanwhile, four-leaf and eight-leaf cabbage seedlings did not respond to vernalization treatment for 40 days in our tested condition (Fig. 1B). This result indicated that cabbage belongs to the plant-vernalization responsive type. To further test whether longer exposure to cold might trigger vernalization response of cabbage seedling, we further exposed four-leaf and eight-leaf cabbage seedlings to 80 day-cold. However, even 80 day-exposed cabbage plants failed to trigger the vernalization-mediated floral transition (Supplementary Fig. S1). This result indicated that seedling developmental stage might be critical for successful vernalization of cabbage, rather than duration of exposure to cold.

Dyanmic Transcriptional Changes Occurred During Vernalization in Cabbage

To understand the vernalization-mediated floral transition of cabbage at the transcriptomic level, we performed RNA-seq analysis to dissect the transcriptomic changes taking place along vernalization time course in early young cabbage (4-week old). Three time points of samples, each with three replicates, were prepared: non-vernalized (NV), 40-day vernalized (V40), and 40-day vernalized and further grown for seven days in warm temperature (After-vernalized; AV). Thus, a total of nine RNA-seq libraries were constructed and sequenced. The result of RNA-seq quality analysis is presented in Supplementary Table S1. A correlation heatmap of the nine RNA-seq data exhibited a close grouping of the samples in the same group, suggesting that the RNA-seq libraries were well prepared along the vernalization time course (Supplementary Fig. S2).

The transcript levels in V40 and AV samples were individually compared to those in the NV samples using the edgeR program. In each comparison, thousands of genes were found to be upregulated or downregulated (Fig. 2A and Supplementary Table S2). A total of 7,784 differentially expressed genes (DEGs) were detected in the V40 samples compared to NV samples, of which 3,669 were upregulated and 4115 were down-regulated in V40 samples (Fig. 2A). The AV samples also displayed thousands of DEGs (Fig. 2A). To identify the overlapping or unique genes in individual comparisons, Venn diagram analysis was performed (Fig. 2B). Among the 7784 DEGs in the V40 samples compared to NV samples, 1240 were detected as genes uniquely expressed in the V40 samples (Fig. 2B). A total of 898 genes were uniquely expressed in the AV samples compared to NV samples (Fig. 2B). This might imply that long-term cold exposure effects those genes and externalize when plants are returned to warm growth condition in the upcoming spring. Also, compared to the NV samples, the expression levels of 951 genes were commonly affected in both groups of vernalized samples (V40 and AV). Interestingly, compared to the V40 samples, a total of 2881 genes were uniquely affected in AV samples, indicating that dynamic transcriptional changes take place both during and after the vernalization. In total, 13,925 genes were differentially modulated in at least one-time point.

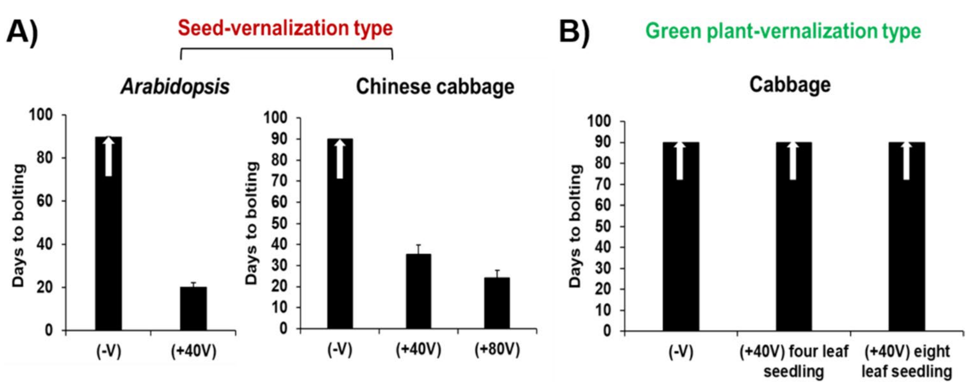


Fig. 1 Measurement of flowering time of two seed-vernalization responsive type plants (*Arabidopsis thaliana* and Chinese cabbage) and a green plant-vernalization responsive type plant (cabbage). **A** Left, Days to flowering of winter-annual *Arabidopsis* seedling at four-leaf stage exposed to non-vernalization (– V) and 40 days vernalization (+40 V). Right, Days to flowering of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) at four-leaf stage exposed to non-vernalization (– V) and 40 days of cold (+40 V). Upward arrows indicate the undetermined flowering time due to the non-flowering up to 90 days after exposure to non-vernalization (– V). **B** Days to flowering of cabbage seedlings (*Brassica oleracea* cv. BN2348) at four-leaf or eight-leaf stage exposed to non-vernalization (– V) and 40 days vernalization (+40 V). Upward arrows indicate the undetermined flowering time due to the non-flowering up to 90 days after exposure to non-vernalization (– V) or 40 days of cold (+40 V)

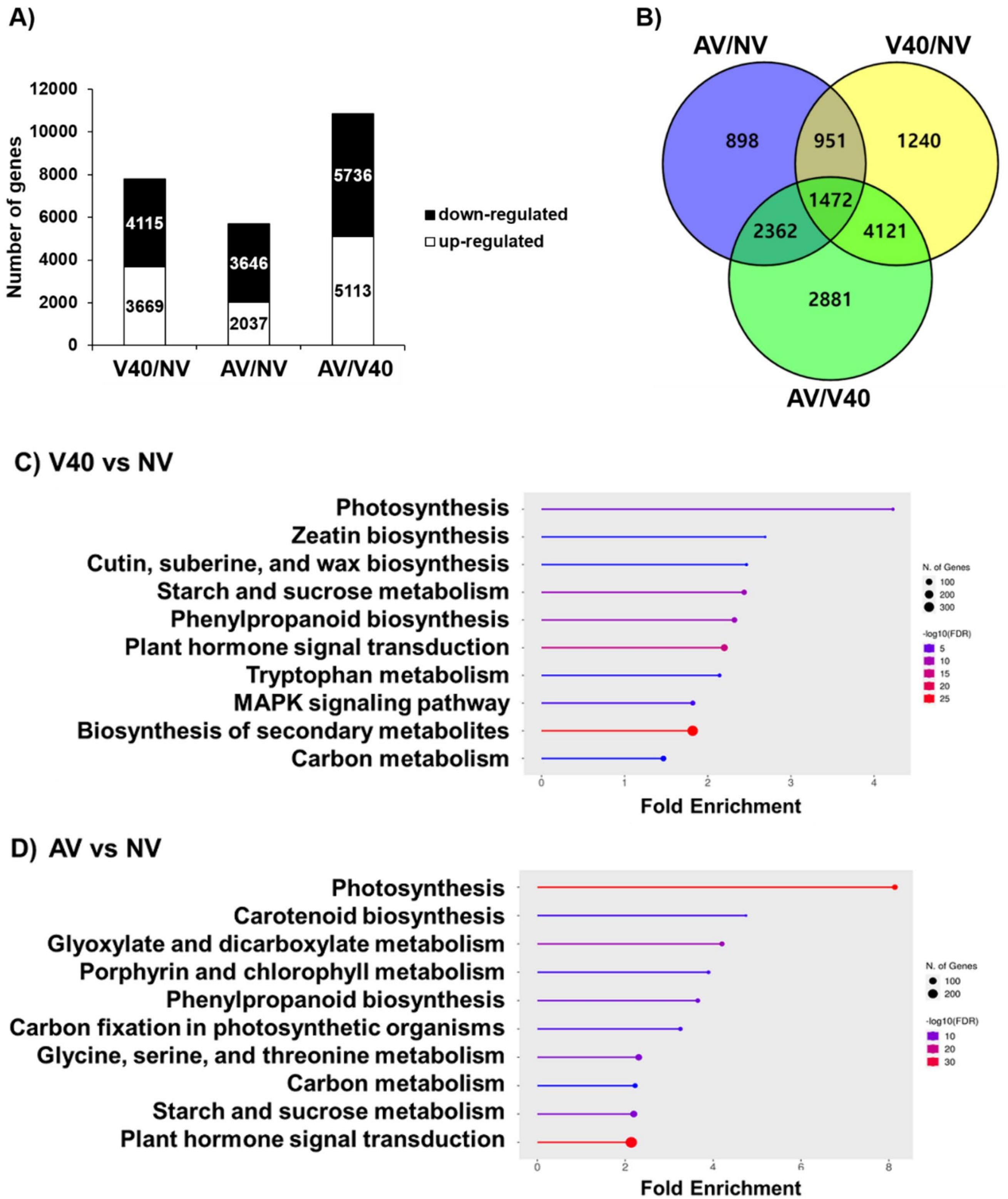


Fig. 2 Transcriptome analysis identifying differentially expressed genes (DEGs) along vernalization time course. **A** Bar graph showing the number of differentially upregulated (white) and downregulated (black) genes for each pair-wise comparison along vernalization time course (NV, V40, and AV). **B** Venn diagram showing the overlapping and unique DEGs for each pair-wise comparison along vernalization time course (NV, V40, and AV). NV, non-vernalized; V40, 40 days of cold; AV, 40 days of cold followed by 7 days of warm temperature. **C**, **D** Gene ontology (GO) analysis of DEGs between vernalized (V40 and AV) samples and non-vernalized (NV) sample. Top 10 functional categories of **C** 40 sample, **D** AV sample compared with the NV sample were presented. *NV* non-vernalized, *V40* 40 day-cold, *AV* 40-day cold followed by 7 days under warm temperature

Dynamic Metabolic Changes take Place in During Vernalization

A gene ontology analysis was performed to identify biological categories enriched in DEGs which were extracted by pair-wise comparison between V40 and NV or AV and NV (Fig. 2C, D). In case of DEGs between V40 and NV, "photosynthesis" category genes were most affected (Fig. 2C), suggesting that photosynthesis might be substantially reduced under low temperature during vernalization. This is consistent with previous reports, which stated that low temperature triggers the repression of genes involved in the "photosynthesis" and "carbohydrate metabolism", thus reducing plant growth (Fowler and Thomashow 2002; Chinnusamy et al. 2007; Xi et al. 2020). Plant hormone (i.e. zeatin) and its related secondary metabolites like "phenylpropanoid" were also significantly affected. In case of DEGs between AV and NV, "photosynthesis" and 'carbohydrate metabolism" category genes were also substantially affected (Fig. 2D). Genes related to the secondary metabolisms like "Carotenoid" and "Phenylpropanoids biosynthesis" were also significantly affected by vernalization. Taken together, these data indicate that vernalization triggers not only developmental transition but also metabolic changes in cabbage.

Gnomic Synteny Analysis Identified Five Cabbage *FLC* Homologs

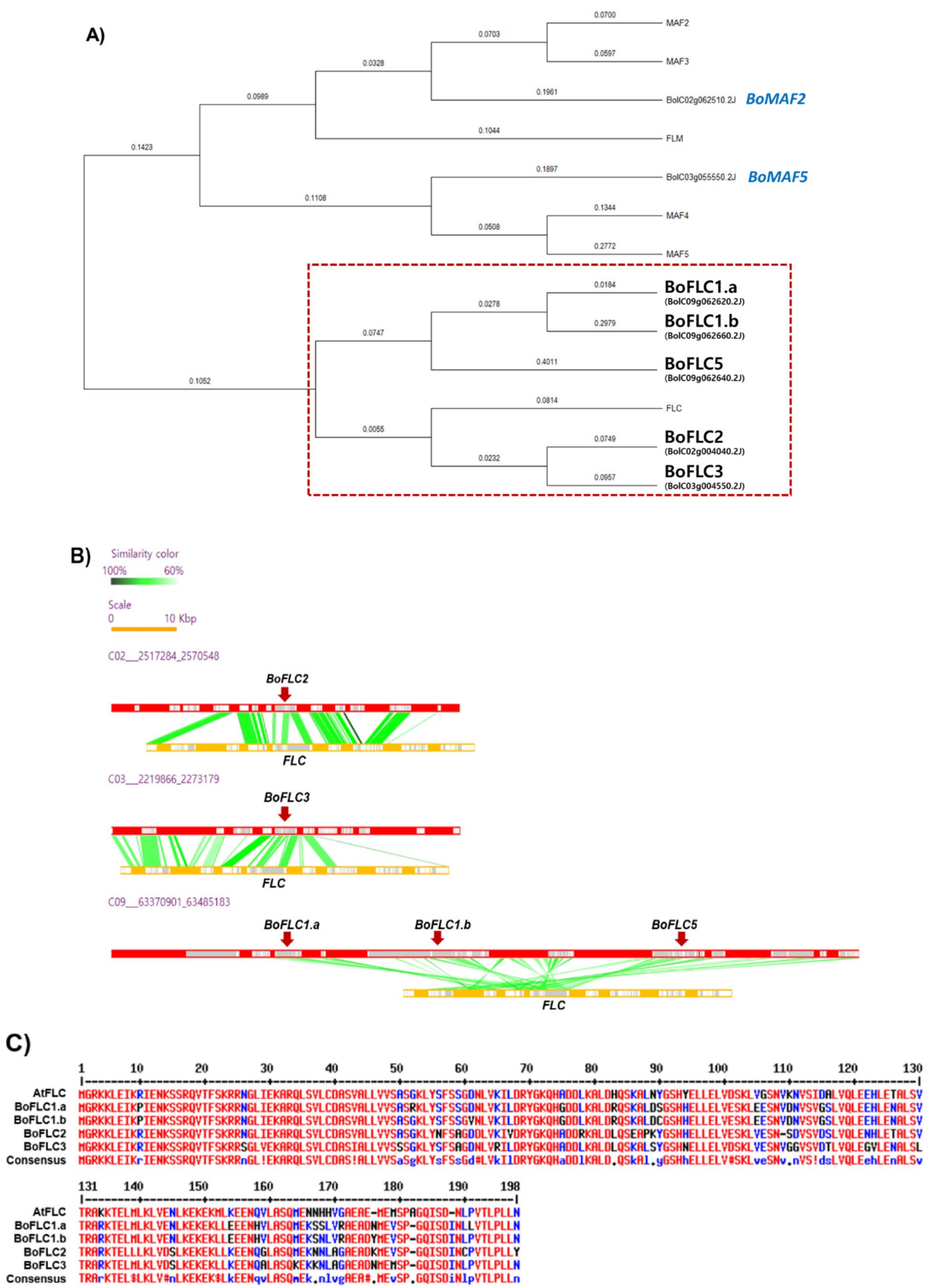
In winter-annual *Arabidopsis* model plants, a MADS-box gene, *FLOWERING LOCUS C* (*FLC*) plays a crucial role in the floral transition. To identify homologs of the *Arabidopsis FLC* gene, we performed phylogenetic analysis using the sequence data of 93 cabbage MADS-box genes and Arabidopsis *FLC* (Fig. S3). As a result, we found five cabbage MADS-box genes clustered together with Arabidopsis *FLC* in the phylogenetic tree and named them as *BoFLC1.a* (BolC09g062620.2J), *BoFLC1.b* (BolC09g062660.2J), *BoFLC2* (BolC02g004040.2J), *BoFLC3* (BolC03g004550.2J), and *BoFLC5* (BolC09g062640.2J) (Fig. 3A and Supplementary Fig. S3). In addition, the syntenic similarity between Arabidopsis *FLC* and cabbage genome was investigated. Similar to the results obtained with the phylogenetic tree, five *BoFLC* homologs were detected (Fig. 3B). Interestingly, while *BoFLC3* and *BoFLC2* were dispersed on two different chromosomes (Chromosome 2 and 3), *BoFLC1.a*, *BoFLC1.b*, and *BoFLC5* were closely clustered together in Chromosome 9 (Fig. S4). All of BoFLCs shared high sequence similarity between them, suggesting that they might act as floral repressors like *Arabidopsis* FLC (Fig. 3C).

Some *FLC* Homologs were not Suppresed by 40 days of Vernalization

All five *BoFLC* homologs were abundantly expressed before vernalization (NV). Therefore, it is likely that all of them contribute to the inhibition of floral transition in the cabbage plant (Fig. 4). When the plants were exposed to low temperature for 40 days (V40), the transcript levels of *BoFLC2* and *BoFLC3* were significantly reduced, whereas those of the other three *BoFLC* homologs (*BoFLC1.a, BoFLC1.b,* and *BoFLC5*) were not affected. When plants were returned to warm growth temperature (AV), the expression of *BoFLC2* and that of *BoFLC3* were a bit de-repressed but maintained at levels lower than those in the NV samples (Fig. 4A, B). To validate the RNA-seq result, we also performed the quantitative RT-PCR analysis on *BoFLC* homologs along vernalizatiome time course (Supplementary Fig. S5). As a result, Similar result to RNA-seq were observed for tested *BoFLC* homologs. These observations suggested that vernalization targets these genes for suppression both during and after the vernalization. Meanwhile, *BoFLC1.a, BoFLC1.b,* and *BoFLC5* were not affected even after vernalization (AV). Accordingly, we postulated that these three cabbage *FLC* homologs might not be direct targets of vernalization. Given the fact that cabbage is the plant vernalization-responsive type, further investigation is needed to clarify whether these three cabbage *FLC* homologs (*BoFLC1.a, BoFLC1.b,* and *BoFLC5*) are responsive to vernalization in different plant stage or cold condition.

Two Cabbage *VIN3* Homologs were Significantly Induced During Vernalization

In Arabidopsis plants, long-term cold exposure induces the expression of a PHD finger domain gene, *VERNALIZATION INSENSITIVE 3* (*VIN3*), which plays an essential role in the vernalization-triggered floral transition through a direct repression of *FLC*. By performing a BLAST search with *Arabidopsis VIN3* sequence, two homologs of *VIN3* were detected in the cabbage genome and named *BoVIN3.C3* (BolC03g013830.2J) and *BoVIN3.C2* (BolC02g015310.2J) (Fig. 4C). The transcription patterns of these *VIN3* homologs were similar to that of *Arabidopsis VIN3*; they were not expressed prior to vernalization, highly induced during vernalization, and then rapidly repressed after vernalization (Fig. 4D, E). Since *Arabidopsis VIN3* plays a pivotal role in the repression of *FLC* upon vernalization, *BoVIN3.C3* and *BoVIN3.C2* might also function in the repression of *BoFLC* homologs in cabbage plants. To validate the RNA-seq result on *BoVIN3* homologs, we conducted the quantitative RT-PCR analysis two *BoVIN3* homologs (Supplementary Fig. S6). As a result, similar result to RNA-seq were observed for *BoVIN3.C3* and *BoVIN3.C2* homologs. In addition, we


A)
MAF2
MAF3
BolC02g062510.2J
BoMAF2
FLM
BolC03g055550.2J
BoMAF5
MAF4
MAF5
BoFLC1.a
(BolC09g062620.2J)
BoFLC1.b
(BolC09g062660.2J)
BoFLC5
(BolC09g062640.2J)
FLC
BoFLC2
(BolC02g004040.2J)
BoFLC3
(BolC03g004550.2J)
0.0700
0.0703
0.0597
0.0328
0.1961
0.0989
0.1044
0.1423
0.1897
0.1108
0.1344
0.0508
0.2772
0.0184
0.0278
0.2979
0.0747
0.4011
0.1052
0.0814
0.0055
0.0749
0.0232
0.0957
B)
Similarity color
100%
60%
Scale
0
10 Kbp
C02__2517284_2570548
BoFLC2
FLC
C03__2219866_2273179
BoFLC3
FLC
C09__63370901_63485183
BoFLC1.a
BoFLC1.b
BoFLC5
FLC
C)
AtFLC
BoFLC1.a
BoFLC1.b
BoFLC2
BoFLC3
Consensus

◂**Fig. 3** Identification of cabbage *FLC* homologs. **A** Result of phylogenic analysis using cabbage and *Arabidopsis FLC* homologs. Five cabbage homologs were found to be clustered with *Arabidopsis FLC* (indicated with a red dotted box). These cabbage *FLC* homologs were named *BoFLC1.a* (BolC09g062620.2J), *BoFLC1.b* (BolC09g062660.2J), *BoFLC2* (BolC02g004040.2J), *BoFLC3* (BolC03g004550.2J), and *BoFLC5* (BolC09g062640.2J). **B** Microsynteny analysis between cabbage and Arabidopsis genome sequence contigs containing *FLC* and its homologs in cabbage. Horizontal red and yellow bars indicate the cabbage and Arabidopsis genome sequence contigs, respectively. White and grey boxes in each bar indicate the exons and introns, respectively. Similar regions in the compared sequences are connected with green lines, and the degree of similarity is depicted using a color gradient as indicated in the first panel (top-left). **C** Result of multiple sequence alignment between *Arabidopsis* FLC and cabbage BoFLC homologs (other than BoFLC5 which is considered as a pseudogene). Identical amino acids among *Arabidopsis* FLC and cabbage BoFLC homologs were indicated with red color letters

also searched cabbage *VIN3-LIKE* (*VIL*) family genes using the BLAST tool in the cabbage genome database and found one cabbage *VIL1* (*BoVIL1*, BolC07g012200.2J) and three *VIN3-LIKE 2* (*BoVIL2.C1*, BolC01g008890.2J; *BoVIL2.C3*, BolC03g082350.2J; *BoVIL2.C7*, BolC07g055120.2J) genes (Fig. 4C). The expression levels of *BoVIL1* and the three *BoVIL2* homologs were not significantly influenced by vernalization, showing a robust expression regardless of vernalization treatment even though some fluctuation in transcript level were observed (Fig. 4F, G). So, it remains unknown whether these BoVIN3 and BoVIL family proteins might share a redundant role in the repression of the five *BoFLC* genes in response to vernalization.

Identification of Genes Showing Similar Expression Patterns to those of *BoVIN3* Transcripts

Total 13,925 DEGs were clustered into twenty groups (Group 1–20) based on distinct transcriptional profiles along the vernalization time course (Fig. 5). Genes in each group are listed in Supplementary Table S3. Among them, Group 9 and 10 exhibited an expression pattern similar to those of *BoVIN3s* (showing induced expression in V40 samples and rapid reduction in AV samples). *BoVIN3.C3* and *BoVIN3.C2* were in Group 9 and Group 10, respectively, in this hierarchical clustering analysis. Generally, transcription factors (TFs) play crucial roles in cellular and developmental processes in plants. Because 1305 (729 + 576) genes in Group 9 and 10 exhibited transcriptional patterns similar to those of *BoVIN3s*, we hypothesized that these groups might contain potential novel transcription factor(s) involved in vernalization-mediated developmental changes in cabbage. Therefore, we extracted genes annotated as TFs from the 'Group 9 and 10'. A total of 96 genes were identified as TFs showing expression patterns similar to those of *BoVIN3s* (Supplementary Table S4). Among them, two floral activator, BoAGL20 homologs (*BoAGL20.1.C4* and *BoAGL20.2.C4*) were also found. Expression patterns of these two *BoAGL20* homologs resembled those of BoVIN3 homologs (showing pattern of substantial increase at V, then decrease at AV time point as shown in the Supplementary Fig. S7B). Besides *BoAGL20* homologs, we also noticed that cabbage *AGL19* homolog (*BoAGL19.C1*) was also included in the Group 9 (Supplementary Table S4). In Arabidopsis, AGL19 was reported to play a positive role in the floral transition. For instance, upregulation of *AGL19* promoted the expression of *FT*, which results in early flowering (Kang et al. 2015). In addition, AGL19 was suggested to act in a *FLC*-independent pathway by elevating the expression of *LFY* and *AP1* to trigger floral transition (Schonrock et al. 2006). Based on this observation, BoAGL19.C1 also might play a positive role in the floral transition of cabbage. Furthermore, we noticed that another MADS-box domain gene, named *BoAGL3.C2* (BolC02g051270.2J.m1) was also found in the Group 9 (Supplementary Table S4). To our knowledge, there has been no report on the functional role of AGL3 related to the flowering in Arabidopsis. It would be one of interesting topics whether BoAGL3.C2 is involved in the floral transition in cabbage.

Expression of two *FT* and Three *SOC1* Homologs were Promoted by Vernalization

In *Arabidopsis*, floral repressor *FLC* inhibits floral transition via the suppression of floral activator genes such as *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*). Using the BLAST search with the sequence of *Arabidopsis FT*, we identified two *BoFT* homologs (named *BoFT.C2* and *BoFT.C6*) showing high levels of sequence similarity to *Arabidopsis FT* (Fig. 6A). Two *BoFT* homologs commonly contained four exons and three introns, the same as *Arabidopsis FT*. Expression levels of *BoFT.C6* were significantly enhanced by vernalization, whereas *BoFT.C2* was merely upregulated throughout the vernalization time course (Fig. 6B, C), suggesting that *BoFT.C6* might play a major role in the floral transition of cabbage plants.

Three *BoSOC1* homologs (named *BoSOC1.C3*, *BoSOC1.1.C4*, and *BoSOC1.2.C4*) were found by using the BLAST search with the sequence of *Arabidopsis SOC1* (Fig. 7A). The expression levels of *BoSOC1.C3*, *BoSOC1.1.C4*, and *BoSOC1.2.C4* were boosted during vernalization (V40) and reduced after vernalization (AV) but still remained higher than those in non-vernalized (NV) samples (Fig. 7B, C). In particular, *BoSOC1.2.C4* exhibited the strongest expression, whereas *BoSOC1.C3* displayed the lowest transcript levels among the *BoSOC1* homologs. Significant upregulation of three *BoSOC1* homologs by vernalization suggested that they play a positive role as floral

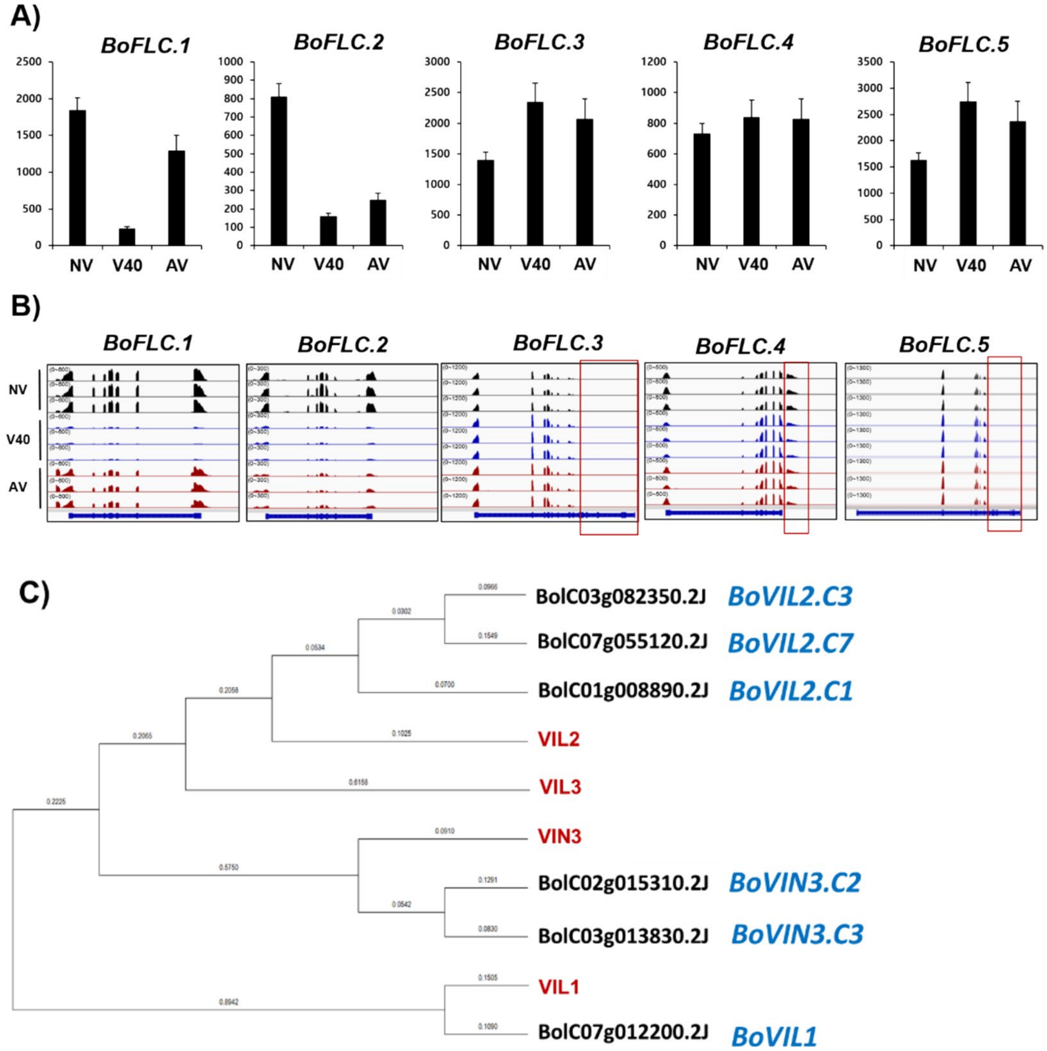


Fig. 4 Transcriptional changes of *BoFLCs* and *BoVIN3* family gene homologs during vernalization time course. **A** Transcript levels of the five *BoFLC* homologs (*BoFLC1.a–5*) during vernalization time course (NV, V40, and AV). Normalized read counts of three biological samples are shown. Significance was statistically determined using one-way analysis of variance (ANOVA) and Tukey's post-hoc test ($p < 0.05$), and indicated with different letters above bars. **B** IGV illustration of normalized RNA-seq read counts of the five *BoFLC* homologs (*BoFLC1.a–5*) along the vernalization time points analyzed (NV, V40, and AV). Transcription of three *BoFLC* homologs (*BoFLC1a*, *BoFLC1.b,* and *BoFLC5*) are not repressed by vernalization, whereas transcript levels of *BoFLC2* and *BoFLC3* are reduced upon the exposure to vernalization. Normalized read counts of three biological replicates are shown for each time point. Mis-annotated regions at *BoFLC1.a* and *BoFLC5* loci are indicated with red boxes. Read coverage normalized by the total number of mapped reads (RPKM) are indicated at the top left corner of each track in parentheses. NV, non-vernalized; V40, 40 days of cold; AV, 40 days of cold followed by 7 days of warm temperature. **C–E** Expression of two *VIN3* homologs (*BoVIN3.C3* and *BoVIN3.C2*) and VIN3-LIKE homologs (*BoVIL1* and *BoVIL2.C1, BoVIL2.C7, and BoVIL2.C3*) along vernalization time points. **C** Phylogenetic tree analysis to identify cabbage homologs of the *Arabidopsis VIN3* and *VIN3-LIKE* (*VIL*) genes. Two homologs of *Arabidopsis VIN3* were named *BoVIN3*.C3 (BolC03g013830.2J) and *BoVIN3*.C2 (BolC02g015310.2J). One homolog of *Arabidopsis VIL1* was named *BoVIL1* (BolC07g012200.2J). Three homologs of *Arabidopsis VIL2* were named *BoVIL2.C1* (BolC01g008890.2J), *BoVIL2.C3* (BolC03g082350.2J), and *BoVIL2.C7* (BolC07g055120.2J). **D** Transcript levels of two *VIN3* homologs (*BoVIN3.C3* and *BoVIN3.C2*) during vernalization time course. Significance was statistically determined using one-way analysis of variance (ANOVA) and Tukey's post-hoc test ($p < 0.05$), and indicated with different letters above bars. **E** IGV illustration of normalized RNA-seq read counts of the two cabbage *BoVIN3* homologs along the vernalization time points (NV, V40, and AV). Normalized read counts of three biological replicates are shown for each time point. Read coverage normalized by the total number of mapped reads (RPKM) are indicated at the top left corner of each track in parentheses. **F** Transcript levels of four cabbage *VIN3-LIKE* (*VIL*) homologs (*BoVIL1*, *BoVIL2.C1*, *BoVIL2.C7*, and *BoVIL2.C3*) during vernalization time course. Significance was statistically determined using one-way analysis of variance (ANOVA) and Tukey's post-hoc test ($p < 0.05$), and indicated with different letters above bars. **G** IGV illustration of normalized RNA-seq read counts of the four cabbage *VIN3-LIKE* (*VIL*) homologs along the vernalization time points (NV, V40, and AV). Normalized read counts of three biological replicates are shown for each time point. Mis-annotated region at *BoVIL2.C3* locus is indicated with red boxes. Read coverage normalized by the total number of mapped reads (RPKM) are indicated at the top left corner of each track in parentheses

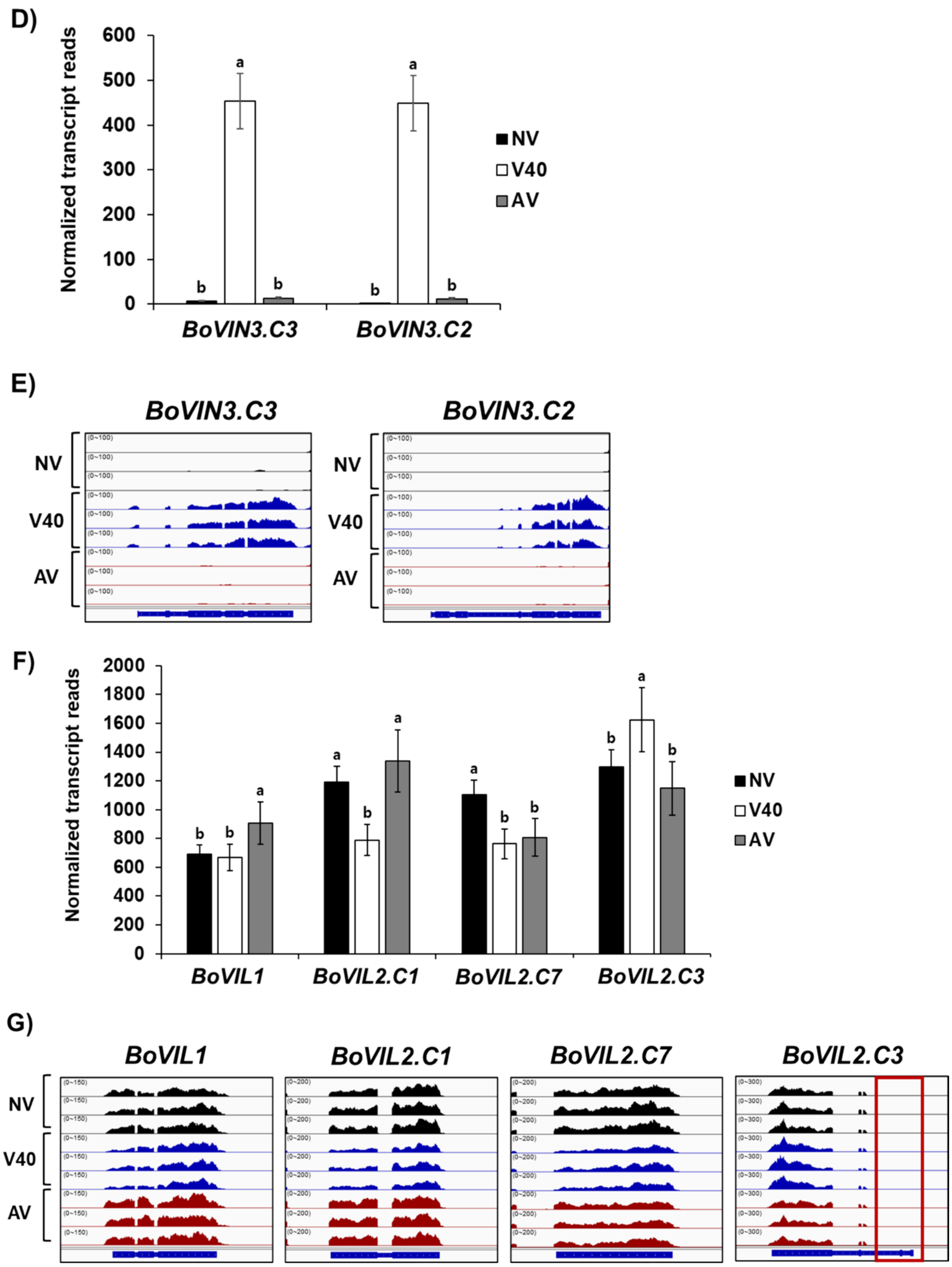


Fig. 4 (continued)

activators in cabbage. To validate the RNA-seq result on *BoFT* and *BoSOC1* homologs, we conducted the quantitative RT-PCR analysis two *BoFTs* and three *BoSOC1* homologs (Supplementary Fig. S7). Resultantly, similar result to RNA-seq were observed for *BoFTs* and *BoSOC1s* homologs along vernalization time course.

It is worth noting that the expression levels of *Arabidopsis FT* and *SOC1* are boosted during vernalization and further enhanced after vernalization. However, this pattern was not observed in either *BoFTs* or *BoSOC1s* in cabbage plants (rather reduced in the AV samples). This discrepancy between *Arabidopsis* and cabbage might be resulted from the incomplete suppression of *BoFLCs* by vernalization in cabbage. Taken together, these results suggested that BoFTs and BoSOC1s might play a positive role in the vernalization-mediated floral transition in cabbage plants.

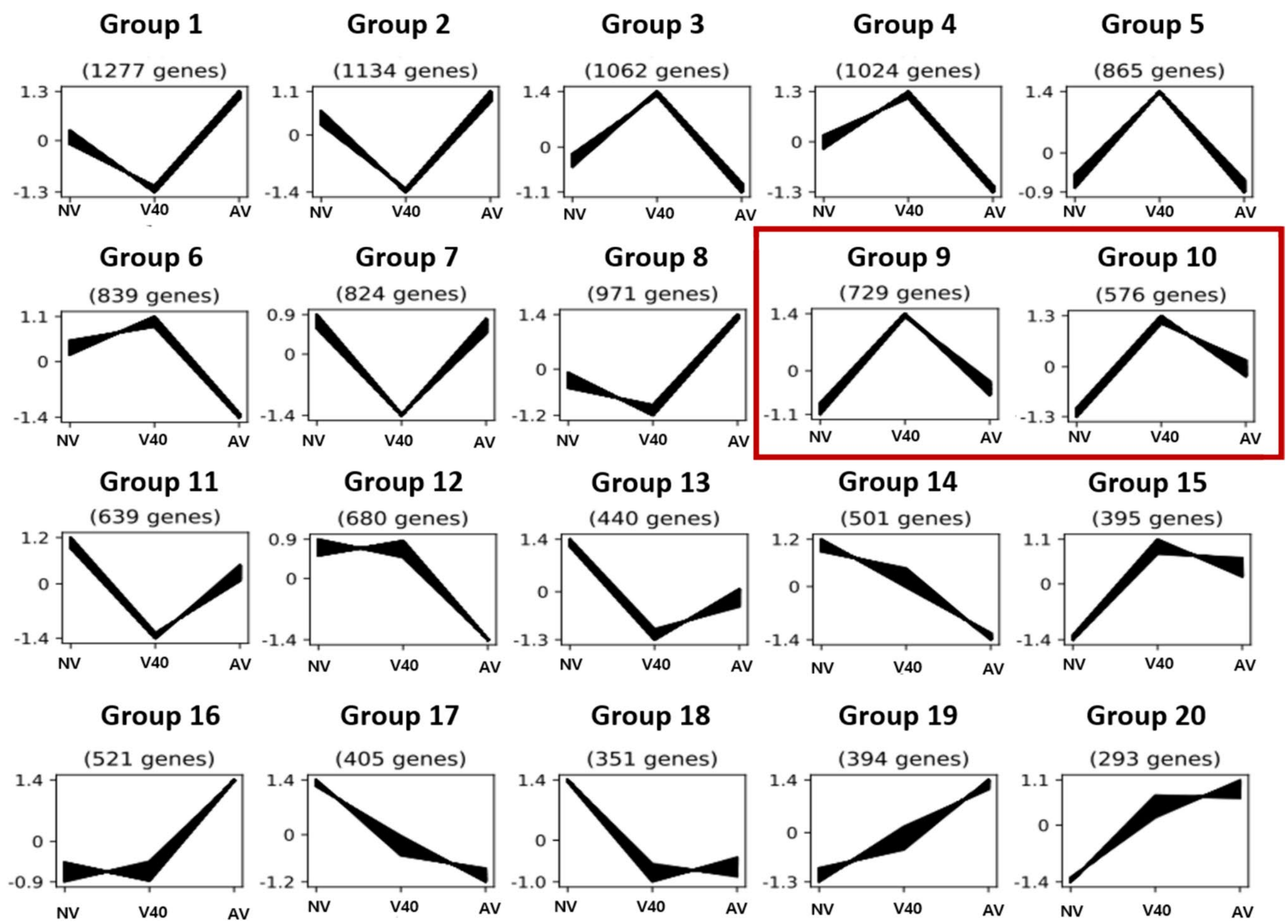


Fig. 5 Hierarchical clustering of differentially expressed genes (DEGs) during vernalization. A total of 20 groups were clustered based on the transcriptional patterns of DEGs during vernalization time course (NV, V40, and AV). Groups 9 and 10, which are indicated with a red box, contain genes showing an expression pattern that is similar to those of *BoVIN3.C3* and *BoVIN3.C2* along vernalization time course. *NV* non-vernalized, *V40* 40 days of cold, *AV* 40 days of cold followed by 7 days of warm temperature

RY/Sph Motif was Commonly Conserved Between *Arabidopsis FLC* and five *BoFLCs*

Vernalization utilizes a histone methyltransferase complex, POLYCOMB REPRESSIVE COMPLEX 2 (PRC2), to suppress *FLC* through the trimethylation of histone H3 at lysine 27 (H3K27me3), a repressive histone mark (De Lucia et al. 2008; Kim and Sung 2013). Recently, it has been demonstrated that a DNA motif called COLD MEMORY ELEMENT (CME) within the first intron of *FLC* is required to recruit the PRC2 complex during vernalization in *Arabidopsis*. CME functions as a recognition site for B3 domain transcription factors such as VIVIPAROUS1/ABI3-LIKE1 (VAL1) and VAL2 in *Arabidopsis*. Because *BoFLC2* and *BoFLC3* were suppressed during vernalization, we analyzed the genomic sequences of five *BoFLC* homologs to determine whether these genes contain CME-like DNA elements. Interestingly, all *BoFLC* homologs were found to contain CME-like elements with RY/Sph motifs(-CATGCA-/-TGCATG-) (Fig. 8A). Even the three *BoFLC* homologs such as *BoFLC1.a, BoFLC1.b,* and *BoFLC5* which did not exhibit a reduced expression in response to vernalization, they commonly had CME-like motifs. In addition, we found that cabbage genome contained two *VAL1* homologs, named *BoVAL1* (BolC01g014720.2J) and *BoVAL2* (BolC07g056290.2J) (Fig. 8B). Their expression levels seemed to be stable throughout the vernalization time points (Fig. 8C, D). Particularly, compared to *BoVAL1*, *BoVAL2* was highly expressed throughout the vernalization time course, suggesting that *BoVAL2* might play a major role in the vernalization-mediated suppression of *BoFLCs* in cabbage. In addition, cabbage *BoVAL1* and *BoVAL2* exhibited a high sequence similarity with *Arabidopsis VAL1* and *VAL2*, indicating that they execute intact functional role as B3 domain transcription factors in cabbage.

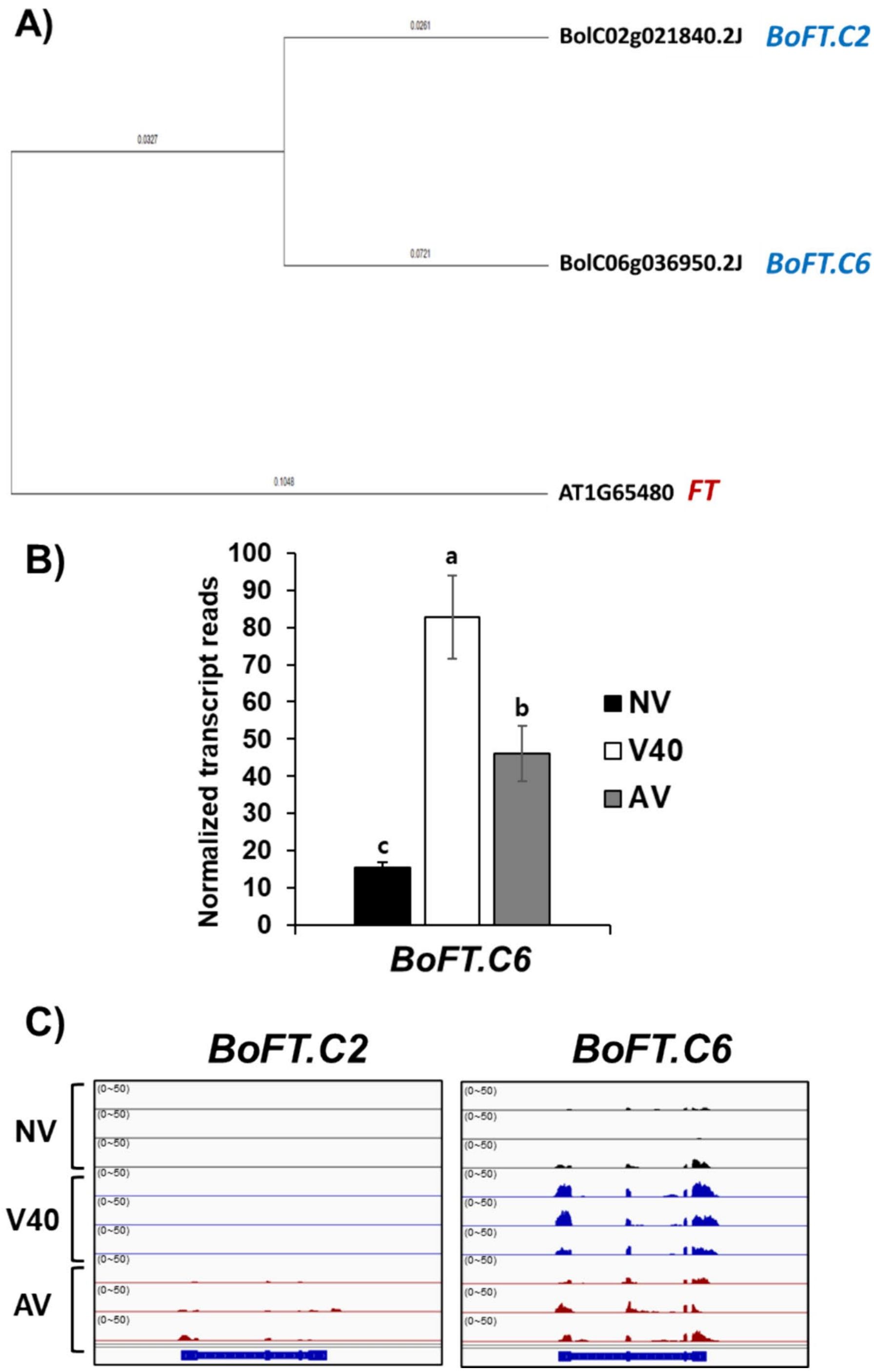


Fig. 6 Expression of cabbage homologs of *Arabidopsis FT*. **A** Phylogenetic tree analysis to identify cabbage homologs of the *Arabidopsis FT* gene. Two homologs, BolC02g021840.2J and BolC06g036950.2J, were closely grouped with *Arabidopsis FT* and named *BoFT.C2* and *BoFT.C6*, respectively. **B** Transcript levels of *BoFT* homolog, *BoFT.C6* during vernalization time course. RNA-seq read counts of *BoFT.C2* were not obtained due to low expression in our transcriptome data. Significance was statistically determined using one-way analysis of variance (ANOVA) and Tukey's post-hoc test ($p < 0.05$), and indicated with different letters above bars. **C** IGV illustrations (upper panel) of normalized RNA-seq read counts of the two cabbage *BoFT* homologs along the vernalization time points (NV, V40, and AV). Normalized read counts of three biological replicates are shown for each time point. Read coverage normalized by the total number of mapped reads (RPKM) are indicated at the top left corner of each track in parentheses

Discussion

Most Brassicaceae family plants, including cabbage, require vernalization to acquire flowering competence. However, each crop has a different preference for vernalization depending on several factors including age, temperature, and duration of vernalization. Early floral transition, particularly in vegetable crops like cabbage, causes a reduction in commercial value. Thus, understanding the molecular details underlying vernalization-mediated flowering is critical for the development of new cultivars with enhanced traits. Prior to vernalization, as in winter, *FLC* homologs are highly expressed and strongly inhibit the floral transition to ensure that vegetable crops such as cabbage grow sufficiently at the vegetative stage. In this study, we investigated the transcriptomic dynamics during vernalization in cabbage (*B. oleracea* var. *capitata* L). We found five *FLC* homologs from the cabbage genome database. Expression of two *BoFLC* homologs

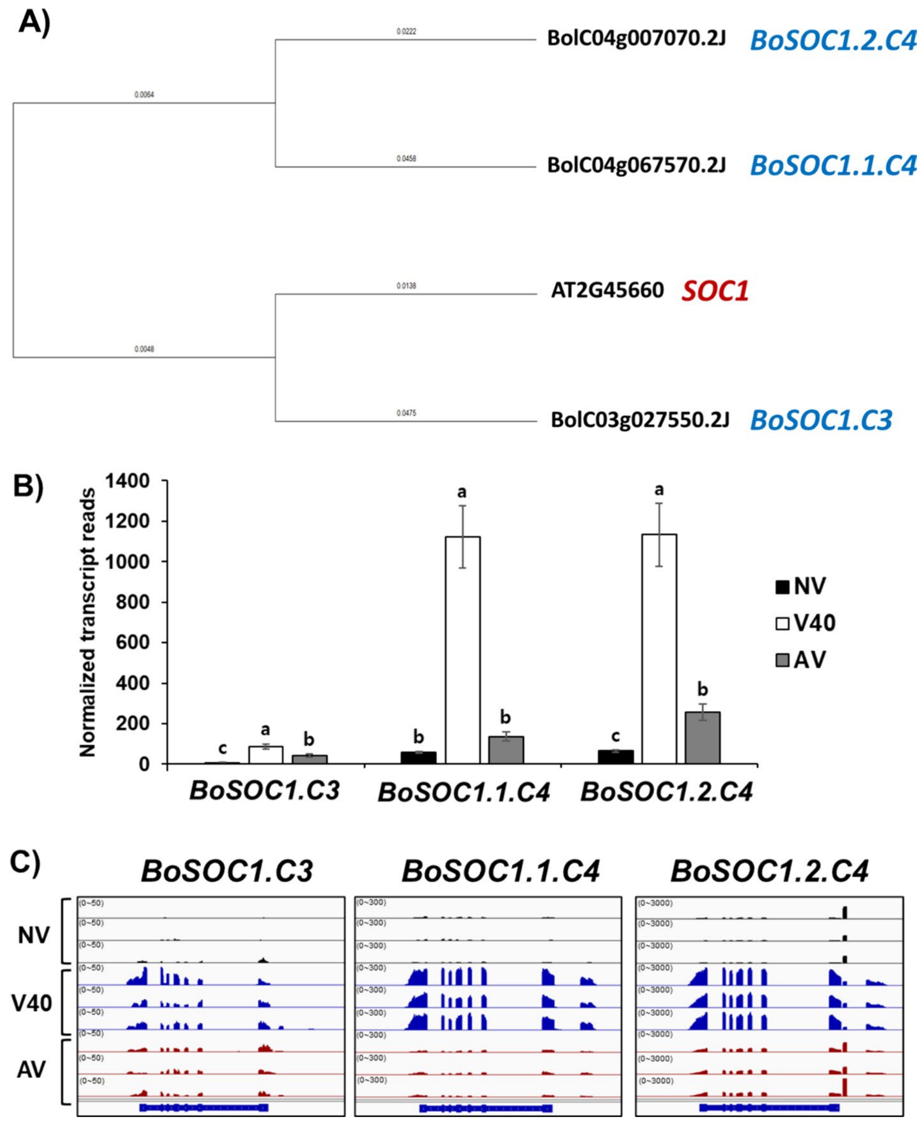


Fig. 7 Expression of cabbage homologs of *Arabidopsis SOC1*. **A** Phylogenetic tree analysis to identify cabbage homologs of the *Arabidopsis SOC1* gene. Three homologs, BolC03g027550.2J, BolC04g067570.2J, and BolC04g007070.2J, were closely grouped with *Arabidopsis SOC1* and named *BoSOC1.C3*, *BoSOC1.1.C4*, and *BoSOC1.2.C4*, respectively. **B** Transcript levels of three *BoSOC1* homologs (*BoSOC1.C3, BoSOC1.1.C4,* and *BoSOC1.2.C4*) during vernalization time course. **C)** IGV illustration of normalized RNA-seq read counts of the three cabbage *BoSOC1* homologs along the vernalization time points (NV, V40, and AV). In particular, *BoSOC1.2.C4* exhibited the strongest expression among the *BoSOC1* homologs, suggesting that *BoSOC1.2.C4* plays a major role as a floral activator in cabbage plant. Normalized read counts of three biological replicates are shown for each time point. Read coverage normalized by the total number of mapped reads (RPKM) are indicated at the top left corner of each track in parentheses. NV, non-vernalized; V40, vernalized for 40 days; AV, vernalized for 40 days followed by further growth for 7 days at warm temperature. Significance was statistically determined using one-way analysis of variance (ANOVA) and Tukey's post-hoc test ($p < 0.05$), and indicated with different letters above bars

(*BoFLC2* and *BoFLC3*) substantially decreased during vernalization, indicative of vernalization responsiveness (Fig. 4A, B). A similar pattern was observed in recent *Brassica rapa* and *Arabidopsis* transcriptome analyses (Kang et al. 2022; Xi et al 2020). However, the other three *BoFLCs* (*BoFLC1.a, BoFLC1.b,* and *BoFLC5*) exhibited stable levels of expression despite the 40-day-long cold exposure, raising the question of why these *BoFLCs* homologs are not responsive to vernalization (Fig. 4A, B). It might be possible that the vegetative age (4-leaf stage) used in this study was insufficient to induce changes in *BoFLC1.a, BoFLC1.b,* and *BoFLC5* expression. Alternatively, the length of cold treatment applied in our study might not be suitable for the optimal vernalization in cabbage plants.

In *Arabidopsis*, it was well demonstrated that transition from vegetative to reproductive stage involves

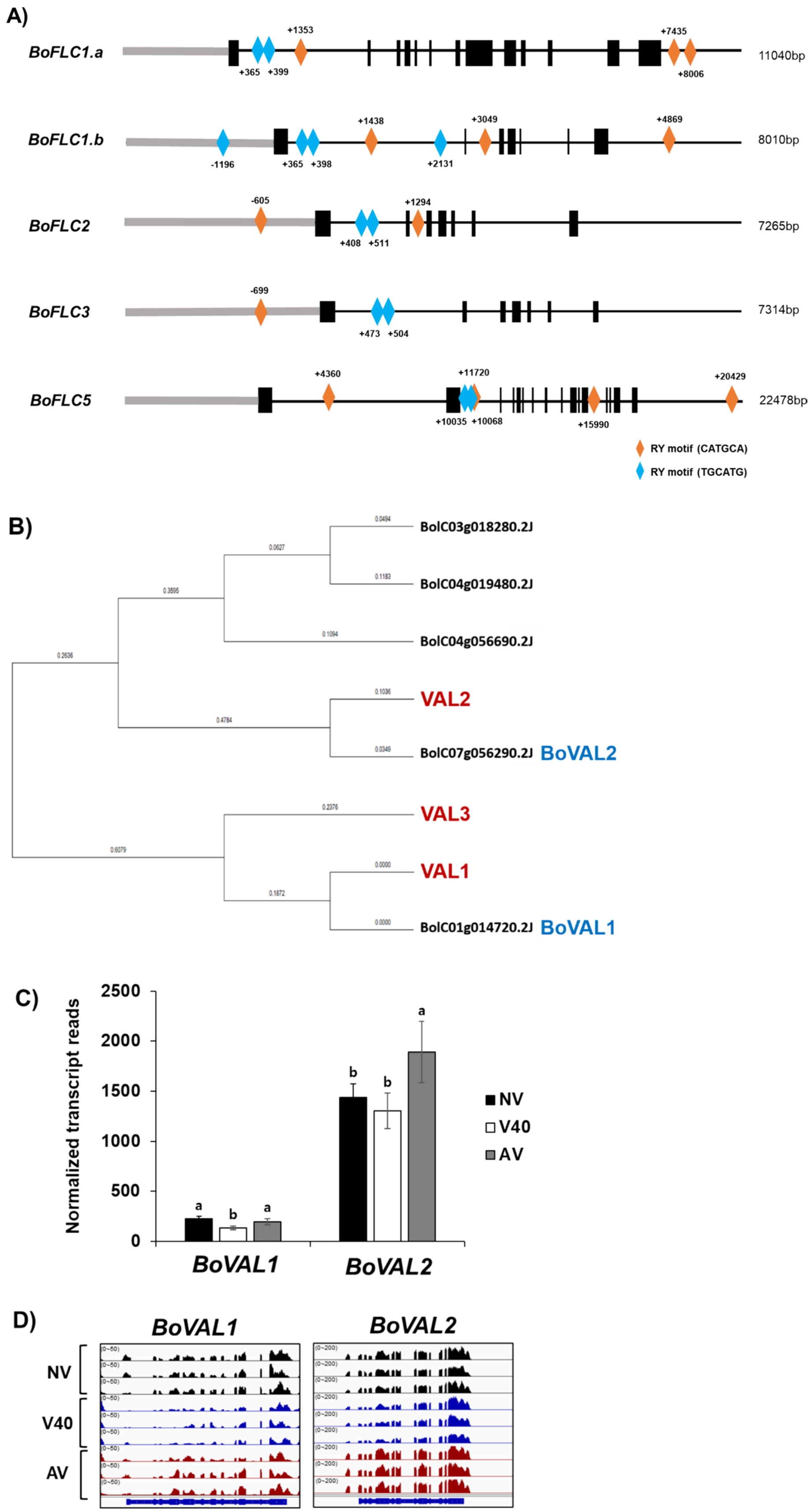


Fig. 8 Identification of CME-like DNA elements in five *BoFLC* homologs. **A** DNA motif analysis of regions possibly involved in the repression of *BoFLCs* via the recruitment of cabbage PRC2 complex. Candidate COLD MEMORY ELEMENT (CME)-like DNA element containing RY/Sph motifs (-CATGCA- or -TGC ATG-) are indicated with orange and blue diamonds. Black boxes and lines presented exons and introns, respectively. Gray lines indicate the promoter region. **B** Phylogenetic tree analysis to identify cabbage homologs of the *Arabidopsis VAL1* and *VAL2*. Two homologs of *VAL1* and *VAL2* were named *BoVAL1* (BolC01g014720.2J) and *BoVAL2* (BolC07g056290.2J), respectively. **C** Normalized transcript levels of *BoVAL1* and *BoVAL2* along vernalization time course (NV, V40, and AV). Transcript levels of *BoVAL2* were much higher than those of *BoVAL1*, suggesting that *BoVAL2* might play a major role in the vernalization-suppression of *BoFLCs* in cabbage. **D** IGV illustration of normalized RNA-seq read counts of the cabbage *BoVAL1 and BoVAL2* homologs along the vernalization time points (NV, V40, and AV). Normalized read counts of three biological replicates are shown for each time point. Read coverage normalized by the total number of mapped reads (RPKM) are indicated at the top left corner of each track in parentheses. NV, non-vernalized; V40, vernalized for 40 days; AV, vernalized for 40 days followed by further growth for 7 days at warm temperature. Significance was statistically determined using one-way analysis of variance (ANOVA) and Tukey's post-hoc test ($p < 0.05$), and indicated with different letters above bars

miRNA-mediated expressional changes on *SPL* genes. For example, miRNA160 play a key role in the inhibition of floral transition via post-transcriptional suppression of *SPL9* and *SPL15*. As plant ages, miRNA160 is gradually decreased and *SPL9/15* is released from the miRNA160-mediated suppression. SPL9/15 acts to boost up the expression of miRNA172 which play a positive role in the activation of floral activators *FT*, *SOC1*, and *AGL24* (Spanudakis and Jackson 2014). It is not known whether miRNAs like miRNA160 and miRNA172 play an important role in cabbage, one of plant vernalization responsive type. Thus, it would be of interest to investigate the discrepancy between the vernalization-responsive group, *BoFLC2* and *BoFLC3*, and the vernalization-insensitive *BoFLC* group, *BoFLC1.a, BoFLC1.b,* and *BoFLC5* in terms of miRNA in cabbage plants in future studies.

In winter-annual *Arabidopsis*, VIN3 expression is induced and plays a crucial role in the repression of *FLC* during vernalization. VIN3 associates with PRC2 complex during vernalization, and a knock-out mutant of *VIN3* is insensitive to vernalization (Sung and Amasino 2004). Both *BoVIN3.C3* and *BoVIN3.C2* were substantially upregulated during vernalization and quickly downregulated when plants were returned to a warm environment, in a manner similar to that of Arabidopsis *VIN3*. However, it is not known whether *BoVIN3s* can redundantly regulate the five *BoFLC* homologs or each *BoVIN3* homolog regulates a subset of *BoFLC* homologs in response to vernalization. Generation of loss-of-function mutants for each *BoVIN3* homolog using CRISPR-Cas9 might help clarify the function of *BoVIN3s* in the cabbage plant.

In summary, we assessed the transcriptomic dynamics in the cabbage plant along the vernalization time course. These results not only contribute to the elucidation of the molecular details of the floral transition of cabbage but also help breeders either build a practical breeding strategy or use biotechnological approaches to create late-flowering cabbage plants.

Materials and Methods

Plant Materials and Vernalization Treatment

Cabbage (*B. oleracea* L. subsp. *capitata*) inbred line 'BN2348' was a gift from the Asia seed company. Seeds were sterilized in 30% bleach solution for 5 min and thoroughly washed with sterile distilled water. Sterilized seeds were placed on half-strength Murashige and Skoog (MS) media. After growing for 4 weeks in warm temperatures under a long-day photoperiod (16 h light, 8 h dark), seedlings reached to 8-leaf stage. These seedling plants were stored in 4 °C for 40 days under a short-day photoperiod (8 h light, 16 h dark). Chinese cabbage (*B. rapa* subsp. *Pekinensis*) cultivar 'Chiifu' and *Arabidopsis thaliana* ecotype 'Columbia-0' were also grown for 4 weeks under the same growth condition as mentioned above. Vernalized seedling cabbage, Chinese cabbage, and *Arabidopsis thaliana* plants were subsequently transferred to a growth room at a warm temperature (22 ± 1 ℃) under a long-day photoperiod (16 h light, 8 h dark).

RNA Extraction

Germinated seedlings were grown in a half-strength MS plate for four weeks at 22 ± 1 °C under a long-day photoperiod (16 h light, 8 h dark) and harvested as the non-vernalized (NV) sample. For vernalization treatment, four-week-old cabbage seedlings were transferred to the refrigerator (4 °C) and stored for 40 days (V40 sample) under a short-day photoperiod (8 h light, 16 h dark). After 40 days of cold exposure, the vernalized samples were returned back to a warm temperature environment (22 ± 1 °C under a long-day photoperiod (16 h light, 8 h dark)) and further grown for seven additional days (AV sample). These time-course seedling samples were used to isolate total RNA using a RNeasy Plant Mini Kit (Qiagen, Germany).

RNA-seq Library Construction and Sequencing

Total RNA was isolated as described above and used to construct RNA-seq libraries using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina Inc., USA) according to the manufacturer's instructions. Libraries were then sequenced on a NovaSeq 6000 system (Insilicogen, South Korea) using the paired-end sequencing protocol. Three biological replicates were prepared for each time point.

RNA-seq Data Analysis

The quality of the RNA-seq reads was evaluated using the FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Low-quality reads were trimmed and filtered out, and only those with more than 90% threshold (Q > 30) were used for mapping. *B. oleracea* reference genome was obtained from the BRAD genome database (http://brassicadb.cn/). Read mapping was performed using the STAR aligner with default parameters (Dobin et al. 2013). Aligned reads were converted to digital counts using FeatureCounts (Liao et al. 2014). Differentially expressed genes (DEGs) were analyzed using edgeR (Robinson et al. 2010) and identified based on a 0.05 p-value and a cutoff of a two-fold difference in expression. Correlation heap map was generated using R packages (ver. 3.6.0). Venn diagram analyses were performed using the Venny webtool (ver. 2.1) (https://bioinfogp.cnb.csic.es/tools/venny/). Aligned reads were then converted to bigwig files for visualization using the Integrative Genomics Viewer program of the Broad Institute (Thorvaldsdóttir et al. 2013).

Clustering Analysis of Co-expressed Genes

The identification of co-expressed gene clusters was performed with Clust software (Abu-Jamous and Kelly 2018). The expressions were normalized by transcript per million (TPM), and the TPM values were used for the clustering analysis. To run Clust software, '—no-optimization' option was used, and '-t' was set as 10. Parsing and visualization of clustering results were performed with in-house python script (https://github.com/managene7/clust_analysis_for_RNA_seqs.git).

Phylogenetic Analysis

Information on amino acid sequences of *Arabidopsis* and cabbage homologs was obtained from the TAIR database (http://www.arabidopsis.org) and the Brassicaceae database (BRAD) (http://brassicadb.cn/), respectively. Protein sequence data of both *Arabidopsis* and cabbage proteins were combined in FASTA format and submitted to the Molecular Evolutionary Genetics Analysis (MEGA) software (Kumar et al. 1994).

Statistical Analysis

One-way analysis of variance (ANOVA) and post-hoc Tukey's test ($p < 0.05$) were used to analyze statistical differences. Data were analyzed using a statistical software package (SAS; version 9.4; SAS Institute Inc., Cary, NC, USA) and presented as the mean ± standard deviation (SD) of three biological replicates.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12374-024-09430-y.

Acknowledgements This research was supported by a research grant from the Chung-Ang University (2023) to DHK. This work was also supported by a grant from the National Research Foundation of Korea (NRF) (No. 2021R1A5A1047822) to DHK, and the National Science Foundation (MCB-2014408) to E.H.

Authors Contributions HM and DHK planned this study; BH prepared plant materials and performed the genetic analysis; HM and MP performed molecular analyses; MP and DHK are involved in the bioinformatic analyses; DHK supervised the study and wrote the manuscript; EH edited the manuscript.

Data Availability NGS sequencing data were deposited to the Gene Expression Omnibus database (accession number, GSE229562).

Declarations

Conflict of Interest The authors declare no conflicts of interest.

References

Abu-Jamous B, Kelly S (2018) Clust: automatic extraction of optimal co-expressed gene clusters from gene expression data. Genome Biol 19(1):172. https://doi.org/10.1186/s13059-018-1536-8

Abuyusuf M, Nath UK, Kim HT, Islam MR, Park JI, Nou IS (2019) Molecular markers based on sequence variation in BoFLC1.C9 for characterizing early- and late-flowering cabbage genotypes. BMC Genet 20:1–11. https://doi.org/10.1186/S12863-019-0740-1/FIGURES/5

Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci 12(10):444–451. https://doi.org/10.1016/j.tplants.2007.07.002

De Lucia F, Crevillen P, Jones AME, Greb T, Dean C (2008) A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of FLC during vernalization. Proc Natl Acad Sci U S A 105:16831–16836

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S et al (2013) STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21. https://doi.org/10.1093/bioinformatics/bts635

Fowler S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14(8):1675–1690. https://doi.org/10.1105/tpc.003483

Hepworth SR, Valverde F, Ravenscroft D, Mouradov A, Coupland G (2002) Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. EMBO J 21(16):4327–4337. https://doi.org/10.1093/emboj/cdf432

Irwin JA, Soumpourou E, Lister C, Ligthart JD, Kennedy S, Dean C (2016) Nucleotide polymorphism affecting FLC expression underpins heading date variation in horticultural brassicas. Plant J 87:597–605. https://doi.org/10.1111/tpj.13221

Kang MJ, Jin HS, Noh YS, Noh B (2015) Repression of flowering under a noninductive photoperiod by the HDA9-AGL19-FT module in Arabidopsis. New Phytol 206(1):281–294. https://doi.org/10.1111/nph.13161

Kang HJ, Nugroho ABD, Park M, Kim JA, Lee SW, Moon H, Choi D, Kim S, Kim D-H (2022) Vernalization regulates flowering genes and modulates glucosinolates biosynthesis in Chinese cabbage. Journal of Plant Biology 65(2):157–173

Kim DH, Sung S (2013) Coordination of the vernalization response through a VIN3 and FLC gene family regulatory network in arabidopsis. Plant Cell 25:454–469. https://doi.org/10.1105/TPC.112.104760

Kinoshita Y, Motoki K, Hosokawa M (2023) Upregulation of tandem duplicated BoFLC1 genes is associated with the non-flowering trait in *Brassica oleracea* var. capitata. Theor Appl Genet 136(3):41. https://doi.org/10.1007/s00122-023-04311-3

Kumar S, Tamura K, Nei M (1994) MEGA: molecular evolutionary genetics analysis software for microcomputers. Bioinformatics 10:189–191. https://doi.org/10.1093/bioinformatics/10.2.189

Lee J, Lee I (2010) Regulation and function of SOC1, a flowering pathway integrator. J Exp Bot 61(9):2247–2254. https://doi.org/10.1093/jxb/erq098

Liao Y, Smyth GK, Shi W (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30:923–930. https://doi.org/10.1093/BIOINFORMATICS/BTT656

Lin SI, Wang JG, Poon SY, Su CL, Wang SS, Chiou TJ (2005) Differential regulation of FLOWERING LOCUS C expression by vernalization in cabbage and Arabidopsis. Plant Physiol 137:1037–1048. https://doi.org/10.1104/PP.104.058974

Lin YR, Lee JY, Tseng MC, Lee CY, Shen CH, Wang CS et al (2018) Subtropical adaptation of a temperate plant (*Brassica oleracea* var. italica) utilizes non-vernalization-responsive QTLs. Sci Rep 8:2–11. https://doi.org/10.1038/s41598-018-31987-1

Moon J, Lee H, Kim M, Lee I (2005) Analysis of flowering pathway integrators in Arabidopsis. Plant Cell Physiol 46(2):292–299. https://doi.org/10.1093/pcp/pci024

Okazaki K, Sakamoto K, Kikuchi R, Saito A, Togashi E, Kuginuki Y et al (2007) Mapping and characterization of FLC homologs and QTL analysis of flowering time in *Brassica oleracea*. Theor Appl Genet 114:595–608. https://doi.org/10.1007/s00122-006-0460-6

Razi H, Howell EC, Newbury HJ, Kearsey MJ (2008) Does sequence polymorphism of FLC paralogues underlie flowering time QTL in *Brassica oleracea*? Theor Appl Genet 116:179–192. https://doi.org/10.1007/s00122-007-0657-3

Ridge S, Brown PH, Hecht V, Driessen RG, Weller JL (2015) The role of BoFLC2 in cauliflower (*Brassica oleracea* var. botrytis L.) reproductive development. J Exp Bot 66:125–135. https://doi.org/10.1093/JXB/ERU408

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26(1):139–140. https://doi.org/10.1093/bioinformatics/btp616

Schonrock N, Bouveret R, Leroy O, Borghi L, Kohler C, Gruissem W, Hennig L (2006) Polycomb-group proteins repress the floral activator AGL19 in the FLC-independent vernalization pathway. Genes Dev 20(12):1667–1678. https://doi.org/10.1101/gad.377206

Spanudakis E, Jackson S (2014) The role of microRNAs in the control of flowering time. J Exp Bot 65(2):365–380. https://doi.org/10.1093/jxb/ert453

Sung S, Amasino RM (2004) Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature 427:159–164. https://doi.org/10.1038/nature02195

Thorvaldsdóttir H, Robinson JT, Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 14:178–192. https://doi.org/10.1093/BIB/BBS017

Wang J, Zhang B, Guo H, Chen L, Han F, Yan C et al (2023) Transcriptome analysis reveals key genes and pathways associated with the regulation of flowering time in cabbage (*Brassica oleracea* L. var. capitata). Plants 12:3413

Xi Y, Park S-R, Kim D-H, Kim E-D, Sung S (2020) Transcriptome and epigenome analyses of vernalization in *Arabidopsis thaliana*. Plant J 103(4):1490–1502

Yoo SK, Chung KS, Kim J, Lee JH, Hong SM, Yoo SJ et al (2005) CONSTANS activates SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 through FLOWERING LOCUS T to promote flowering in Arabidopsis. Plant Physiol 139:770–778. https://doi.org/10.1104/PP.105.066928