Gene Expression Analysis in Response to Vernalization in Chinese Cabbage (*Brassica rapa* L.)

Ayasha Akter^{1,2*}, Junji Miyazaki³, Daniel J. Shea⁴, Namiko Nishida¹, Satoko Takada¹, Naomi Miyaji¹, Hasan Mehraj¹, Motoki Shimizu⁵, Md. Asad-ud Doullah⁶, Takeshi Takasaki-Yasuda¹, Keiichi Okazaki⁴ and Ryo Fujimoto¹

¹Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan

²Department of Horticulture, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

³Agriculture Victoria Research Division, Department of Jobs, Precincts and Regions, AgriBioscience, Bundoora, Victoria 3083, Australia

⁴Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan

⁵Iwate Biotechnology Research Center, Kitakami 024-0003, Japan

⁶Department of Plant Pathology and Seed Science, Faculty of Agriculture, Sylhet Agricultural University, Sylhet-3100, Bangladesh

Chinese cabbage (*Brassica rapa* L. var. *pekinensis*) is an economically and agriculturally significant leafy vegetable that is extensively cultivated throughout the world. Vernalization is exposure to prolonged cold that alters gene expression and accelerates a transition from the vegetative to reproductive phase. Premature bolting caused by exposure to cold inhibits head production and reduces the yield of Chinese cabbage, and developing a late bolting line is important for breeding. Therefore, it is critical to identify the genes showing differential expression patterns during cold treatment in Chinese cabbage. However, there are few studies on the transcriptome profiling of different durations of cold treatments in Chinese cabbage. Here, we analyzed the gene expression profiles in a Chinese cabbage inbred line, RJKB-T24, given different durations of cold treatments using RNA sequencing. Differentially expressed genes (DEGs) between non-vernalized and vernalized samples tended to be downregulated, and some genes involved in the flowering pathway (including *BrFLC* and *BrMAF* genes) were downregulated following cold treatment. Functional enrichment analysis indicated that some DEGs were involved in the stress response and hormone signaling pathways. For genes involved in the FRI-containing complex, a known activator of *FLC* in *Arabidopsis thaliana*, only *BrFRL1* showed changes in expression. In contrast to *A. thaliana*, *BrVIP* and *BrVRN* genes showed different expression patterns between paralogs during cold treatment, suggesting that Chinese cabbage's flowering pathway is somehow different and more complex than in *A. thaliana*. These outcomes provide significant insights into the genetic control of bolting and flowering that occur during the vernalization of Chinese cabbage.

Key Words: differentially expressed genes, flowering pathway, RNA-sequencing, transcriptome.

Introduction

Chinese cabbage (*Brassica rapa* L. var. *pekinensis*) is an important vegetable that contributes to human health

as a source of beneficial dietary fibers and vitamins. *B. rapa* is the first species in the genus *Brassica* to be sequenced, and a double haploid (DH) line of Chinese cabbage, chiifu-401-42, was examined by sequencing (Wang et al., 2011). Whole genome sequencing of this line showed that *B. rapa* experienced a whole genome triplication (WGT) after speciation between the genera *Brassica* and *Arabidopsis* (Cheng et al., 2012; Wang et al., 2011). Vernalization refers to prolonged exposure to cold temperature, and is usually required to facilitate flowering in Brassicaceae. Because Chinese cabbage can sense low temperatures during seed germination (seed-vernalization-responsive type), it sometimes un-

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* Corresponding author (E-mail: aakterhort@bau.edu.bd).

dergoes early flowering/bolting due to low temperatures that occur during production in the fall (Shea et al., 2018; Su et al., 2018). Premature bolting leads to a decline in commercial quality and a loss of market value. When breeding Chinese cabbage cultivars, a high bolting resistance is preferred (Shea et al., 2018).

How vernalization promotes flowering in *Arabidopsis thaliana* has been extensively studied. Two genes, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), play vital roles in the flowering process in *A. thaliana*. *FRI* encodes a novel protein with two coiled-coil domains and is an activator of *FLC* (Johanson et al., 2000). *FLC* encodes a MADS-box transcription factor and acts as a suppressor of flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). During cold treatment, *FLC* expression is decreased and maintained at the same level even after a return to warm conditions. *VERNALIZATION INSENSITIVE 3* (*VIN3*), *VERNALIZATION1* (*VRN1*), *VRN2*, and *VRN5* have been identified as regulatory genes of *FLC* expression associated with vernalization (Greb et al., 2007; He and Amasino, 2005; Wood et al., 2006). At the beginning of cold treatment, *VIN3* is activated (Sung and Amasino, 2004), while expression of *VRN1* and *VRN2* does not change during cold treatment (Gendall et al., 2001; Levy et al., 2002). Five MADS-box genes, *MADS AFFECTING FLOWERING1* (*MAF1*) to *MAF5*, belong to the MADS-box protein family containing *FLC* (Alvarez-Buylla et al., 2000; Ratcliffe et al., 2001, 2003) and act as floral repressors (Kim and Sung, 2010; Ratcliffe et al., 2001; Scortecci et al., 2001, 2003; Sheldon et al., 2009). *MAF* genes show temperature-dependent changes in expression; *MAF1*–*MAF4* are downregulated by vernalization, whereas *MAF5* is upregulated (Ratcliffe et al., 2001, 2003). *MAFs* also directly interact with each other and produce complexes that regulate the genes related to floral transition such as *FLOWERING LOCUS T* (*FT*) (Gu et al., 2013).

Transcriptional repression of *FLC* during prolonged cold exposure is associated with changes in histone modifications from active marks (trimethylation of lysine 4 in histone H3 (H3K4me3) or H3K36me3) to a repressive mark (H3K27me3), initiating a conformational change from euchromatin to heterochromatin (Dennis and Peacock, 2007; Groszmann et al., 2011). This is mediated by Polycomb repressive complex 2 (PRC2) components including the Su(z)12 homologue, *VRN2*, and two plant homeodomain (PHD) finger proteins, *VRN5* and *VIN3* (Bastow et al., 2004; Birve et al., 2001; De Lucia et al., 2008; Gendall et al., 2001; Sung and Amasino, 2004; Wood et al., 2006). Upon being returned to warm conditions after prolonged cold exposure, the PHD–PRC2 spreads across the *FLC* locus with spreading H3K27me3 marks, and the expression of *FLC* is epigenetically silenced (Angel et al., 2011; Finnegan and Dennis, 2007). *VRN1* encodes a DNA-

binding protein containing two plant-specific B3 domains, and is required for the increase in H3K27me3 at the *FLC* locus during prolonged cold treatment (Levy et al., 2002; Sheldon et al., 2006).

In Chinese cabbage, the reduced transcription of *FLC* could be an important factor in premature bolting (Itabashi et al., 2018; Shea et al., 2018; Su et al., 2018; Takada et al., 2019). There are four *FLC* paralogs in *B. rapa* and at least three of them act as floral repressors (Kawanabe et al., 2016; Kim et al., 2007; Schranz et al., 2002; Takada et al., 2019). All *FLC* paralogs were downregulated by prolonged cold treatment, and an increased level of H3K27me3 was observed around the first exon in all *FLC* paralogs in Chinese cabbage (Akter et al., 2019; Kawanabe et al., 2016). Following a return to normal temperature growth conditions, H3K27me3 spreads along all four *BrFLC* paralogs providing stable repression of the gene (Akter et al., 2019; Takada et al., 2019). Many QTL analyses using populations derived from the crossing of parents with two different flowering times showed co-localization of QTLs and *FLC* genes, suggesting that the different function of *FLC* alleles between lines results in variations in flowering time (Kakizaki et al., 2011; Kitamoto et al., 2014; Kole et al., 2001; Li et al., 2009; Lou et al., 2007; Osborn et al., 1997; Schranz et al., 2002; Xiao et al., 2013; Zhao et al., 2010).

Innovations in sequencing technology have enabled us to examine the transcriptome by RNA-sequencing (RNA-seq), providing high accuracy, a wider detection range of transcription, and high reproducibility (Ansorge, 2009; Mortazavi et al., 2008; Saeki et al., 2016). Using this technology, transcriptional changes in response to many kinds of abiotic or biotic stresses have been examined in the genus *Brassica* (Long et al., 2015; Miyaji et al., 2017; Wang et al., 2016; Xing et al., 2016; Yong et al., 2014; Zou et al., 2013). This method has also been applied to the detection of transcriptional changes in Brassicaceae following vernalization in pak-choi (*B. rapa* var. *chinensis*) and radish (*Raphanus sativus*) (Liu et al., 2017; Sun et al., 2015).

There is no report examining the transcriptome associated with different durations of vernalization treatments in Chinese cabbage. In this study, we analyzed the transcriptome during vernalization by RNA-seq to discover genes that respond to cold treatment and to identify the metabolic pathways involved in the cold response in Chinese cabbage. Our study provides an important opportunity to facilitate the research on vernalization mechanisms in *B. rapa*.

Materials and Methods

Plant materials and growth conditions

A Chinese cabbage inbred line, RJKB-T24, was used as the plant material (Kawamura et al., 2016). Seeds were surface-sterilized and grown on agar solidified Murashige and Skoog (MS) medium with 1% (w/v)

sucrose under long day (LD) conditions (16 h light) at 22°C. For cold treatment, 14-day seedlings on MS plates were treated for two (2V), four (4V), or six (6V) weeks at 4°C under the LD conditions (16 h light), or six weeks at 4°C and then seven days in normal growth conditions (6V7N). RJKB-T24 did not flower without four weeks of cold treatment even if more than 100 days passed after sowing. When four weeks of cold treatment was applied to 14-day seedlings, flowering occurred 66 days after cold treatment. These results indicate that RJKB-T24 requires vernalization for flowering.

RNA extraction and RT-PCR/qPCR

First and second leaves with (2V, 4V, 6V, 6V7N) or without (NV) cold treatments were used for isolation of total RNAs using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The leaves from three individual plants in each condition were harvested as biological replicates. The cDNA was synthesized from 500 ng total RNAs using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO Co., Ltd., Osaka, JAPAN). The cDNA was PCR amplified using Quick Taq® HS DyeMix (TOYOBO) for RT-PCR. Prior to quantitative real-time RT-PCR (RT-qPCR), the specificity of the primer set for each gene was first tested by electrophoresis of PCR-amplified products using QuickTaq® HS DyeMix (TOYOBO) on a 2.0% agarose gel in which single products were observed. Absence of genomic DNA contamination was confirmed by PCR of a no RT control using the *BrACTIN* (*BrACT*) gene (Fujimoto et al., 2006). PCR was performed using the following conditions; 1 cycle of 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s. Primer sequences used for RT-PCR are shown in Table S1.

RT-qPCR was performed by using LightCycler 96 (Roche Diagnostics International Ltd., Switzerland) and cDNA was amplified using FastStart Essential DNA Green Master (Roche). PCR conditions were 95°C for 10 min followed by 55 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s, and a melting program (60°C to 95°C at 0.1°C/s). After amplification cycles, each reaction was subjected to melt temperature analysis to confirm single amplified products. The expression level of each gene relative to *BrACT* was automatically calculated using automatic CQ calling according to the manufacturer's instructions (Roche). Data presented are the means and standard error (s.e.) calculated from three biological and experimental replications. Primer sequences used for RT-qPCR are shown in Table S1. Dunnett's test was performed for statistical analysis.

RNA-sequencing

RNA-sequencing (RNA-seq) was performed using first and second leaves with (2V, 4V, 6V, 6V7N) or without (NV) cold treatments. A sequence library was

prepared as described previously (Nagano et al., 2015). RNA-seq was performed with three replicates of each condition using Illumina Hiseq2000 (single-end sequencing, 50 bp). Sequence data have been submitted to the DDBJ database (<http://www.ddbj.nig.ac.jp>) under accession number DRA009156.

The quality of the RNA-seq libraries was evaluated using the FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). After QC, filtered reads were used for further analysis. Tophat2 (Kim et al., 2013) with the default parameters was used to align RNA-seq reads against the *B. rapa* reference genome downloaded from the Ensemble Genome database (ftp://ftp.ensemblgenomes.org/pub/plants/release-32/fasta/brassica_rapa/dna/Brassica_rapa.IVFCAASv1.dna.toplevel.fa). The levels of gene expression were scored by fragments per kilo-base per million (FPKM) using cufflinks, and cuffdiff was used for identification of differentially expressed genes (DEGs) with and without cold treatments (Trapnell et al., 2012). Analysis for enrichment of gene functional ontology terms was completed using the gene ontology (GO) tool agriGO (Du et al., 2010) according to methods we previously described (Shimizu et al., 2014). Statistical tests for enrichment of functional terms used the hypergeometric test and false discovery rate (FDR) correction for multiple testing to a level of 5% FDR.

We validated the RNA-seq data by RT-qPCR using 25 genes, and 23 of the 25 genes showed similar expression patterns to RNA-seq data (FPKM) (Fig. S1; Table S2).

Results

Identification of differentially expressed genes between vernalized and non-vernalized samples

We performed the RNA-seq in leaves with and without cold treatments in an inbred line of Chinese cabbage, RJKB-T24 that requires vernalization for flowering. Samples with three different durations of cold treatments, two (2V), four (4V), or six (6V) weeks, and after a return to warm conditions for seven days following six weeks of cold treatment (6V7N) were used. Total reads and mapped reads on the reference genome are shown in Table S3. The differentially expressed genes (DEGs) between non-vernalized (NV) and vernalized samples (2V, 4V, 6V, 6V7N) were identified by Tophat/cuffdiff at 95% confidence. A total of 3,787 (2V vs. NV), 3,543 (4V vs. NV), 2,630 (6V vs. NV), and 3,896 (6V7N vs. NV) DEGs were identified, including 1,169, 1,291, 980, and 946 upregulated, and 2,618, 2,252, 1,650, and 2,950 downregulated, genes in 2V, 4V, 6V, or 6V7N, respectively (Table 1).

Comparing DEGs between the different cold treatment conditions, 345 upregulated and 1,222 downregulated genes overlapped between 2V/NV and 4V/NV, and the number of overlapped upregulated genes decreased between 2V/NV and 6V/NV and between

2V/NV and 6V7N/NV (Fig. 1). The number of overlapped downregulated genes decreased between 2V/NV and 6V/NV, whereas it increased between 2V/NV and 6V7N/NV (Fig. 1). The numbers of overlapped upregulated and downregulated genes between 4V/NV and 6V/NV were 322 and 934, respectively, and 277 upregulated and 1,207 downregulated genes overlapped between 4V/NV and 6V7N/NV (Fig. 1). Between 6V/NV and 6V7N/NV, 211 upregulated and 936 downregulated genes overlapped (Fig. 1). There were 128 upregulated and 48 downregulated genes in three conditions (2V/NV, 4V/NV, and 6V/NV), and 646 upregulated and 430 downregulated genes were found in four conditions (2V/NV, 4V/NV, 6V/NV, and 6V7N/NV) (Table 1).

Transcriptional changes in flowering pathway genes following cold treatment

Genes involved in flowering pathways in *B. rapa* have been identified (Wang et al., 2017), and 89 genes mainly involved in vernalization and autonomous pathways were selected in this study (Table S4). In our RNA-seq data, 29 out of 89 genes showed differential expression in vernalized samples compared with non-vernalized samples for at least one cold treatment condition (Fig. 2; Table S4). More genes showed downregulation than upregulation (Fig. 2; Table S4).

Expression levels of *BrVIN3* (Bra020445) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*BrSOC1*; Bra004928 and Bra039324) rose with longer cold treatment durations (Fig. 2). From RT-qPCR results, the expression of *BrVIN3*s decreased on return to normal temperature, while the increased expression of *BrSOC1*s was maintained (Fig. 3A). Three *BrFLC* paralogs (*BrFLC1*/Bra009055, *BrFLC2*/Bra028599, *BrFLC3*/Bra006051) and three of five *BrMAF* genes (Bra024350, Bra024351, Bra031888) were downregulated by cold treatment (Fig. 2), and repression of the expression levels was maintained after return to normal

temperature (Fig. 2). Downregulation of the total amount of four *BrFLC* genes and three *BrMAF* genes was also confirmed by RT-qPCR (Fig. 3B).

From the RNA-seq data, we identified that *BrUBC1* (Bra026833), *BrSEF* (Bra006766), *BrFRL1* (Bra008624), and one *BrVRN1* (Bra022376) were upregulated by cold treatment (Figs. 2 and S1). *BrLD* (Bra018540), *BrUBC2* (Bra026582), one *VERNALIZATION INDEPENDENCE 4* (*BrVIP4*) paralog (Bra029332), *BrVIP5* (Bra031459), *BrVIP6/BrELF8* (Bra013162), and two of three *BrVRN1* paralogs

Fig. 1. The total number of overlapped genes under different vernalization conditions. NV, non-vernalized; 2V, two weeks of cold treatment; 4V, four weeks of cold treatment; 6V, six weeks of cold treatment; 6V7N, six weeks of cold treatment followed by seven days under normal growth conditions.

Table 1. Number of differentially expressed genes in vernalized and non-vernalized samples.

	Upregulated in vernalized samples	Downregulated in vernalized samples
1. 2V vs. NV (2V/NV)	1,169	2,618
2. 4V vs. NV (4V/NV)	1,291	2,252
3. 6V vs. NV (6V/NV)	980	1,650
4. 6V7N vs. NV (6V7N/NV)	946	2,950
Overlapped genes (1, 2, 3)	128	48
Overlapped genes (1, 2, 3, 4)	646	430

NV, non-vernalized samples.

2V, samples with two weeks of cold treatment.

4V, samples with four weeks of cold treatment.

6V, samples with six weeks of cold treatment.

6V7N, six weeks of cold treatment followed by seven days under normal growth conditions.

Fig. 2. Heat map of the expression levels in genes involved in the flowering pathway showing upregulation or downregulation by cold treatment. Expression levels (FPKM) higher or lower than the median are shown. NV, non-vernalized; 2V, two weeks of cold treatment; 4V, four weeks of cold treatment; 6V, six weeks of cold treatment; 6V7N, six weeks of cold treatment followed by seven days under normal growth conditions.

Fig. 3. Validation of the expression levels by RT-qPCR for selected genes involved in the flowering pathway. The expression levels of each gene relative to *BrACTIN* (*BrACT*) were calculated, and the y-axis shows the ratio of vernalized samples (2V, 4V, 6V, 6V7N) compared with non-vernalized samples (NV). Data presented are the average and standard error (s.e.) from three biological and experimental replications. Statistical tests between non-vernalized and vernalized samples are shown (Dunnett's test, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). NV, non-vernalized; 2V, two weeks of cold treatment; 4V, four weeks of cold treatment; 6V, six weeks of cold treatment; 6V7N, six weeks of cold treatment followed by seven days under normal growth conditions.

(Bra037544, Bra001729) showed downregulation by cold treatment (Fig. 2). In addition, we found opposite expression patterns between *BrVIP4* paralogs (Fig. 3C) and between *BrVRN1* paralogs (Fig. 3D).

Functional annotation of differentially expressed genes after vernalization

To identify the functions of DEGs, GO enrichment analysis was conducted. The upregulated and downregulated genes at different durations of vernalization (2V, 4V, 6V, and 6V7N) were categorized into the GO cellular component (CC), GO molecular function (MF), and GO biological process (BP). In upregulated genes in 2V, 4V, 6V, and 6V7N, 25, 64, 93, and 22 categories were overrepresented ($P < 0.0001$, FDR < 0.05), respectively (Tables S5–S8). Twelve categories were overrepresented in upregulated genes in 2V, 4V, and 6V, and six categories were overrepresented in upregulated genes in all four conditions (Fig. S2). In downregulated genes in 2V, 4V, 6V, and 6V7N, 344, 372, 241, and 377 categories were overrepresented ($P < 0.0001$, FDR < 0.05), respectively (Tables S5–S8). Ten categories were overrepresented in downregulated genes in 2V, 4V, and 6V, and 73 categories were overrepresented in downregulated genes in all four conditions (Fig. S2).

The categories related to stress response such as

'Response to cold' or response to plant hormone stimulation such as 'Response to jasmonic acid stimulus', 'Response to ethylene stimulus', and 'Response to salicylic acid stimulus' tended to be overrepresented in both upregulated and downregulated genes by cold treatment (Figs. 4 and S2). The category 'Response to heat' tended to be overrepresented in upregulated genes, while the categories of 'Small molecule metabolic process' and 'Defense response' tended to be overrepresented in downregulated genes (Figs. 4 and S2). The category 'Regulation of transcription' was overrepresented in upregulated genes, especially at 4V (Fig. 4).

We performed RT-qPCR to examine the expression of six genes related to stress response. The expression level of *HEAT SHOCK TRANSCRIPTION FACTOR B2A* (*BrHSFB2A*) increased with cumulative cold treatment time, and its expression decreased when it returned to normal temperature (Fig. 5). The expression level of *HEAT SHOCK PROTEIN 70* (*BrHSP70*) increased with cumulative cold treatment time, and its expression was maintained when it returned to normal temperature (Fig. 5). *COLD REGULATED 15B* (*BrCOR15B*) showed rapid induction of its expression at low temperature and this expression was undetectable when it returned to normal temperature

Fig. 4. GO enrichment analysis using upregulated or downregulated genes by cold treatment. The Log10 score of the p -value shows overrepresentation. The heat map shows overrepresentation higher or lower than the median. All selected categories belong to the GO biological process (BP). NV, non-vernalized; 2V, two weeks of cold treatment; 4V, four weeks of cold treatment; 6V, six weeks of cold treatment; 6V7N, six weeks of cold treatment followed by seven days under normal growth conditions.

Fig. 5. The expression patterns of six genes related to stress response measured by RT-qPCR. The expression level of each gene relative to *BrACTIN* (*BrACT*) was calculated. The relative expression levels of vernalized samples (2V, 4V, 6V, 6V7N) compared with non-vernalized samples (NV) are shown. Data presented are the average and standard error (s.e.) from three biological and experimental replications. Dunnett's test was performed to compare the expression levels between non-vernalized and vernalized samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NV, non-vernalized; 2V, two weeks of cold treatment; 4V, four weeks of cold treatment; 6V, six weeks of cold treatment; 6V7N, six weeks of cold treatment followed by seven days under normal growth conditions.

(Fig. 5). Expression levels of *INDOLE-3-ACETIC ACID INDUCIBLE 28* (*BrIAA28*), *IAA-LEUCINE RESISTANT (ILR)-LIKE GENE 6* (*BrILL6*), and *BrMYB28* were cumulatively downregulated by cold treatment, and repression of the expression was maintained after return to normal temperature (Fig. 5).

Discussion

Vernalization is one of the major pathways for flowering regulation, and there are variations in flowering times and vernalization requirements among different lines and cultivars of Chinese cabbage (Su et al., 2018). Previous research revealed that *BrFLC* gene(s) is a key factor for vernalization in Chinese cabbage (Itabashi

et al., 2018; Shea et al., 2018; Su et al., 2018; Takada et al., 2019). However, there is limited research showing how the regulators of *BrFLC* genes react in response to vernalization or the transcriptional response at the whole genome level during prolonged cold treatment in Chinese cabbage.

In this study, we analyzed the transcriptome using first and second leaves before and after vernalization, and differentially expressed genes tended to be downregulated by cold treatment. During cold treatments, plant growth was slow, but leaf size gradually increased according to the low temperature period. In 6V7N, the plant growth rate returned to normal when the temperature was returned to that of normal growth conditions, and the leaf size of 6V7N was larger than NV. Comparing the expression levels in vernalized samples (2V, 4V, 6V, 6V7N) and non-vernalized samples (NV), it was suggested that to some extent genes whose expression levels changed depending on the different growth stages were involved, especially in 6V7N. A cold responsive gene, *BrCOR15B*, was highly induced in response to cold treatment, but rapidly returned to previous expression levels upon return to normal growth conditions. *BrSOC1* genes were upregulated during cold treatment. RT-qPCR data showed that the *BrSOC1* expression level in 6V7N was still higher than NV, while RNA-seq data showed similar expression levels between 6V7N and NV. The expression patterns of *BrSOC1* in previous reports in *B. rapa* were similar to the RT-qPCR results (Akter et al., 2019; Kawanabe et al., 2016), suggesting that the RT-qPCR results represent the correct expression pattern. The *BrVIN3* gene was upregulated and *BrFLC* genes were downregulated during cold treatment, consistent with previous studies in *A. thaliana* and *B. rapa* (Deng et al., 2011; Jung et al., 2018; Kawanabe et al., 2016; Sheldon et al., 2000, 2006). These results indicate that we could identify genes for which the expression level was changed by cold treatment.

GO categories related to stress or stimulus tended to be overrepresented in both upregulated and downregulated genes by cold treatment, but some GO categories showed a difference between upregulated and downregulated genes. The category 'Response to heat' was overrepresented in upregulated genes, and we showed that expression levels of *BrHSFB2A* and *BrHSP70* increased during cold treatment. It has already been shown that *Hsfs* and *HSPs*, which were first described as factors that function under high temperature conditions, are induced at low temperatures (Swindell et al., 2007). These results suggest that upregulation of genes categorized as 'Response to heat' play a role in cold stress response. The category 'Regulation of transcription' was overrepresented in upregulated genes after four weeks of cold treatment, suggesting that some transcription factors are activated when a sufficient period of cold is applied. In contrast, the categories

'Response to biotic stimulus' and 'Defense response' were overrepresented in downregulated genes. Some studies have reported the cross-talk between biotic stress response and the transition to flowering (Chin et al., 2013; Lyons et al., 2015). The genes categorized as "response to plant hormone stimulation" tended to show altered the expression levels following cold treatment. We found the downregulation of genes was involved in auxin signaling, and previous studies showed that a decreased IAA hormone level was detected after vernalization in *B. rapa* (Huang et al., 2018a). Although we could not distinguish between genes involved in the vernalization response promoting flowering and the physical cold response, some genes identified in this study could be involved in the vernalization response or the transition from the vegetative to reproductive phase (e.g., *BrSOC1*).

In *A. thaliana*, regulators of *AtFLC* have been identified (Kim and Sung, 2014). The FRI containing complex (FRI-C) including *AtFRI*, *AtFRL1*, *AtFES1*, *AtSUF4*, and *AtFLX*, and the PAF1 complex including *AtVIP4*, *AtVIP5*, *AtELF7*, and *AtELF8* are known as activators of *AtFLC* expression (Kim and Sung, 2014; Shea et al., 2018). In this study, *BrFRL1* expression levels were increased with cumulative cold treatment time, while other genes involved in FRI-C did not show any change in their expression levels during cold treatment. Expressions of *BrFLC* genes were downregulated with cumulative cold treatment time; therefore, the upregulation *BrFRL1* could be independent from the downregulation of *BrFLC* genes during cold treatment. Genes involved in the PAF1 complex showed a change in expression levels during cold treatment in Chinese cabbage. *BrVIP3* and one of two *BrVIP4* genes showed upregulation by cold treatment, while one of two *BrVIP4*, *BrVIP5*, and *BrVIP6* genes showed downregulation by cold treatment. In *A. thaliana*, vernalization-associated changes in mRNA for *AtVIP4*, *AtVIP5*, or *AtVIP6* have not been observed (Oh et al., 2004; Zhang and van Nocker, 2002). If the PAF1 complex is also an activator of *BrFLC* genes in Chinese cabbage, the downregulation of the genes involved in the PAF1 complex by vernalization could result in the downregulation of *BrFLC* genes.

Genes involved in autonomous pathways or components of PHD-PRC2 are known as repressors of *AtFLC* expression in *A. thaliana* (Kim and Sung, 2014; Michaels and Amasino, 2001). Expression levels of *AtVRN1* and *AtVRN2* are not affected by vernalization in *A. thaliana* (Gendall et al., 2001; Levy et al., 2002). However, in this study, expression levels of *BrVRN1* genes changed by cold treatment in Chinese cabbage, and the expression patterns observed during cold treatment were different among the three paralogous genes; two *BrVRN1* genes were downregulated and one *BrVRN1* gene was upregulated. Upregulation of *BrVRN1* by 20 days of cold treatment was also ob-

served in pak choi (Sun et al., 2015). These results suggest that, unlike in *A. thaliana*, it may be necessary to examine the possibility that the suppression of *BrFLC* expression is dependent on the upregulation of *BrVRN1* by cold in *B. rapa*.

Five *BrMAF* genes were identified in the reference genome of *B. rapa*, but an orthologous relationship between *BrMAF* genes in *B. rapa* and *AtMAF1-5* in *A. thaliana* was not able to be determined (Cheng et al., 2012). Two *BcMAF* genes in pak choi, *BcMAF1* (homologous gene, Bra031884, blastp, $3e^{-61}$) and *BcMAF2* (homologous gene, Bra031888, blastp, $2e^{-74}$), have been shown to act as floral repressors (Huang et al., 2018b, 2019). In this study, we showed that three of five *BrMAF* genes were downregulated with cumulative cold treatment time, suggesting that downregulation of *BrMAF* genes is important for promotion of flowering by vernalization. Further research will be required to clarify how much the *BrMAF* genes contribute to the promotion of flowering by vernalization in comparison with *BrFLC* genes.

In this study, we identified differentially expressed genes following cold treatment and some genes involved in flowering pathways showed different expression patterns between Chinese cabbage and *A. thaliana*. In addition, in genes involved in the flowering pathways, we found a difference in expression patterns between paralogous genes during cold treatment in Chinese cabbage, resulting in a more complicated flowering pathway in Chinese cabbage than in *A. thaliana*. The findings in *A. thaliana* can mostly be applied to the molecular mechanism(s) of vernalization in Chinese cabbage, but there are some differences compared to *A. thaliana*; for example, cold-induced noncoding RNAs were not found in Chinese cabbage (Li et al., 2016; Shea et al., 2019). Further analyses of the genes found in this study will be required to identify the genetic factors involved in vernalization and the precise molecular mechanism of vernalization in Chinese cabbage.

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