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Expression of Flowering-Related Genes in Two Inbred Lines of Chinese Cabbage

Hyun-Seung Jang, Yong-Pyo Lim, Yoonkang Hur*

Genome Research Center, Chungnam National University, Daejon 305-764, Korea

Abstract

Expression of 43 flowering-related genes was examined in two inbred lines of Chinese cabbage, Chiifu and Kenshin, under different photoperiod, vernalization and flower development stages. The floral genes cloned by RT-PCR with degenerated primers showed high homology with *Arabidopsis* counterparts. Genes in two inbred lines, *TOC*, *CRY1*, *CO*, *RGAL* and *GAI*, were highly expressed under all tested conditions. However, expression of three genes was regulated by particular experimental conditions. The expression of *LHY* gene was predominant in Chiifu under the short-day conditions, whereas the expression of *RGAL* gene was influenced by vernalization in both inbred lines. Besides, the expression of *NAP* gene was induced by vernalization only in Chiifu. Most of the flower identity-related genes were expressed during flower development. The transcript level for several genes was not detected in this experiment.

Key words: *Brassica rapa ssp. pekinensis*, flowering genes, vernalization, photoperiod

Introduction

Flowering, the transition from vegetative growth to reproductive development or flowering stage, is controlled by both environmental and developmental signals. During the past decade, significant progress has been made towards understanding the molecular basis of flowering in *Arabidopsis*. The control of flowering and genes associated with the mechanism have recently been reviewed by several workers (Reeves and Coupland 2000; Samach and Coupland 2000; Araki 2001; Mouradov et al. 2002; Simpson and Dean 2002).

In *Arabidopsis*, floral transition is promoted by exposure to low temperatures and growth under long-day conditions. As a result of physiological, genetic, and molecular analysis of *Arabidopsis* mutants altering in flowering time, the existence of four floral promotion pathways was proposed in *Arabidopsis* (Mouradov et al. 2002; Simpson and Dean, 2002); Photoperiod response pathway, vernalization response pathway, autonomous pathway and GA (gibberellin) pathway. Mutations in the genes, such as *CONSTANS* (*CO*), *GIGANTEA* (*GI*), and *FLOWERING LOCUS T* (*FT*), which are involved in the long-day pathway caused late flowering under long-days but did not delay flowering under short-days when compared to the wild type. On the contrary, mutations in genes such as *FCA*, *LUMINIDEPENDENS* (*LD*), *FVE*, and *FPA* that are involved in the autonomous pathway caused late flowering under both long-days and short-days in comparison to the wild type. In addition, mutants of the genes involved in autonomous pathway showed a strong response to vernalization, and a prolonged cold treatment accelerated flowering, suggesting that the vernalization and autonomous pathways merge at some point (Koornneef et al. 1991, 1998).

Recently, a great progress in the mechanism of flowering has been achieved in vernalization and circadian control. *FLOWRING LOCUS C* (*FLC*), which encodes a MADS-box protein, appeared to function as a repressor of flowering in *Arabidopsis* and its level decreased upon vernalization (Sheldon et al. 2000). The vernalization caused change in DNA methylation status, resulting in the repression of FLC expression. Circadian clock-related genes, *ZTL* (*ZEITLUPE*) and *FKF1* (*FLAVIN-BINDING, KELCH REPEAT, F-BOX*), were responsible for connections between the photoperiodic light signaling and circadian clock control (Mizoguchi and Coupland

* Corresponding author, E-mail: ykhur@cnu.ac.kr
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2000; Imaizumi et al. 2003).

The genus *Brassica* includes many important vegetable crops, such as broccoli, cabbage, Chinese cabbage, cauliflower, mustard, rape, kale and turnip. Although these *Brassica* species have served as good model plants to study self-incompatibility (Takasaki et al. 2000; Suzuki et al. 2003), very little is known about the floral genes. Schranz et al. (2002) reported that flowering time gene, *FLC*, from Chinese cabbage had similar function with *Arabidopsis* homolog with respect to modulate flowering time. Control of flowering time in Chinese cabbage, a very important vegetable crop in Asia, is very important for efficient seed production and for cultivation during the summer season. Therefore, isolation of floral genes and elucidation of the flowering mechanism in Chinese cabbage are an urgent project.

In this study, we report isolation of floral genes, identified in *Arabidopsis*, from two different lines, Chiifu and Kenshin, of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) and examined their expression characteristics in relation to photoperiod, vernalization and floral organ development.

Materials and Method

Plant materials

Brassica rapa ssp. *pekinensis* inbred lines, Chiifu (A line) and Kenshin (B line), were used in this experiments. All stages of Chinese plants, from germinating seedling to flowering plant, were harvested to isolate flowering-related genes by RT-PCR. Vernalization was given by incubating the germinated seedlings in petri dishes to the 4°C for 20 days. Hypocotyls including cotyledons were harvested and frozen in liquid nitrogen. Exposure to photoperiod was either a long-day (LD, 16 h light/8 h dark) or a short-day (SD, 8 h light/16 h dark). Plants grown in growth chambers (23 ± 2°C) were exposed to two photoperiods from cotyledon stage to 45 days of day after germination (DAF). Plant leaves were collected at the end of light or dark period.

RNA isolation and reverse transcription

Plant samples were ground in a mortar with liquid nitrogen and 100-500 mg of the fine powder were used to extract total RNAs using TRIZOL reagent (Invitorgen). The total RNAs were further cleaned up using RNeasy Plant Mini Kit (Qiagen). Quantity and quality of the RNAs were measured by a UV spectrophotometer and gel electrophoresis.

After treatment of DNase-I, the reverse transcription was carried out with 1-2 μg of total RNAs using a cDNA synthesis kit with oligo (dT) as primers (Promega). The synthesized cDNAs were used for further experiments.

Gene cloning and sequence analysis

To clone flowering-realted genes from Chinese cabbage, degenerated primers were prepared on the basis of sequence information from known genes (Table 1). Using sense and antisense primers (10 pmol), each gene was amplified by PCR using 1 μL cDNA as the template DNA in 20 μL reaction mixture. The PCR reaction was carried out with an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s - at 55°C for 40 s - at 72°C for 60 s, and final extension at 72°C for 7 min. The PCR products were ligated to pGEM-T Easy vectors (Promega) and transformed into *E. coli* JM109 cells. The subcloned DNAs were sequenced with a dye-terminator cycle sequencing kit (PerkinElmer) and a DNA sequencer (ABI 3700, PerkinElmer). Sequence data were analyzed with known sequences in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) with BLAST search program.

Membrane preparation and hybridization

To amplify the flowering-related genes, PCR for the recombinant plasmid DNA was performed with same primers as described in Table 1. The PCR condition was 2 min at 94°C, 30 cycles of 94°C for 15 s - 52°C for 30 s - 72°C for 60 s, and 5 min at 72°C for the final extension. Equal amounts (200 ng) of the amplified and denatured DNA were spotted onto the nylon membrane (Schleicher & Schuell) using Bio-Dot Microfiltration apparatus (Bio-Rad). The membrane was dried and incubated in UV cross-linker for 150 s. β-tubulin gene was spotted on the membrane as control.

Probes were prepared by reverse transcription of RNA samples as follows. Total RNAs (5 μg) freshly extracted from Chinese cabbage samples were subjected to reverse transcription in the presence of radio-labeled dCTP with oligo-dT primers as described as in the manufacturer's manual (SuperScript RT-System, GIBCO BRL). The probe was purifiled using a QIAquick spin column (Qiagen).

Hybridization was carried out by incubating the membrane in a solution (5X SSC, 5X Denhardt's reagent, 0.1% SDS, 50 % formaminde and 100 μg /mL denatured fragmented ssDNA) for overnight at 42°C. After hybridization, the membrane was washed twice at room temperature with 6 X SSC/0.5% SDS for 15 min, twice at 37°C with 1 X SSC/0.5% SDS for 15 min and once at 65°C with 0.1 X SSC/0.1% SDS for 30 min. The signal was detected with Personal Molecular Imager FX (Bio-Rad) and analyzed with Quantity One software.

Table 1. List of genes cloned and degenerated primers used.

Classification		Gene	Sense primer (5' → 3')	Antisense primer (5' → 3')
Light Signal	Circadian rhythm	*ELF3*	ATGGCNGCNACNTGYGARAT	TTNCCYTCNARRCARTCCCARTA
		TOC1	CACACAGAAGTAGAGGGACC	GGCGTTTCCTATTCACATAC
		LHY	GACCTCAACTGTGGACAAC	TGAAGGTGTATTTGAGCCAC
		CCA1	CAAGGACCTCAGACTTATCCGATGCA	TCCATTGGCAGCCCACCAA
		FKF1	CACGTAATTGGGATACAGGT	GACCGATAAAGAATGCCC
		ZTL	CACGGGTATTAGAGACTGT	GATCCATTGTGAAGACATC
		GI	CTGAAAATATTGAGGCGAAT	CAGTGAAACAACGGAGAAAG
	Photoperiod	*PHYA*	ATGCAYCCWAGGTCATCDTTYAA	GCTKGCAAGYTGYAGRAARCARAA
		PHYB	TTYGGKYTDCAGYTVAAYATGGA	GGARTGWATYGCATCCATYTC
		CRY1	GAGCAATGCTGATAAGTTAC	GGAGAAGAAACTTCACAG
		CRY2	CTWMGGVTDGAGGAYAAYCCRGC	GCRTCNARNARNGTRTCCCARAA
		CO	GANRTYCAYTCYGCMAAYCC	TCYCTKTAYCTSAGVACYCTNGC
		FT	TTGTGTGTTACGAAAATCC	CATCACCGTTCGTTACTCGT
		FWA	GCAACATTTGTGGTAAGGCA	CCTTTAGCTCTTGAAGCCT
Autonomous Pathway		*FCA*	ATGAAYGGTCCNCCAGAYAGAGT	AGAACRTTTCCRTGYTGYTCRAA
		LD	TGGTACTGCAGTTGCTAGAT	TCATGTTTCCTTGATGCTT
		FRI	TTAGCTGCGTTTTCAGTT	TACCACGATCAGGCATTAGA
		FLC	GAGACGAAATGGTCTCATGG	CGGAGGAGGAGTTGTAGAG
Vernaiization Pathway		*VRN2*	GCTTGCTGGACAATTTGAG	CTCTGATCGCTCAGCAGATA
Gibberellin Pathway		*GA4*	CGTTGAAGAGTACGAGGAAC	TCACCGATTGGTATAGAGGC
		GA5	CATCTCCTGAGGAAGAAGAC	GCATCGCAGAAGTAATCTTG
		GAI	CACTTCACCGCRAAYCARGC	TARTGCARMGACTCRGTRAA
		SPY	TCAAGAGTCAGCGTTACTGG	CTAAAAAGCCTTAAAACCCT
		RGAL	GTGATTAAAGAAGAAGCTGC	GAAATACGGTACCGTTATGG
Flowering Time		*TFL*	GCAAAGAGGTGGTGAGCTAT	CAGGGAGACCAAGATCATAC
		LFY	GACGAACCAAGTATTCAGGT	CCAAACTAGAAACGCAAG
		AP1	GTAGTTTCTTATTGGGGGTC	CAAGAGCAACTTCAGCAT
		CAL	GGTAGGGTTGAATTGAAGAG	GAGTACTCGAACAATTTGCC
		UFO	GAGTCAGTTGCCACCAATAT	TTCCAGTAACTTCATAGGAC
		CLF	GATATCAATGGAAATATGGT	AGCTTCAGGTTTTTTGGCCCA
Meristem Identity		*AG*	CGYCAAGTYACYTTYTGTAA	TCAABTTCYCTTTTYTGCAT
		AP2	CACCCATCAGTTCTTCCCTG	CGGTAAAACGTAACACCTCT
		AP3	GCTGAAGATCCTCACTATG	TAGATAGACAATGATGGCAC
		PI	GGAGATGGCTATAGCAAGCG	GTTCGAAACGTTTAAGCACA
		LUG	AATCAGTTGGCTGAYATGGA	AGCGTCCATACYTTNACCAT
		HUA2	GAGGGCTGTTTGTATCTACG	TTTGAAACCACTGTTAGCCT
		CRC	TTGGGATACCATTGAAGAGA	GTGAGGCTAACATGGCCTTG
		SPT	TTCTTCTTCCTCGGTTTACG	CGCTTTCACAGTCATACTCA
		NAP	TGAATGGGTACTGTGTAGGA	TCATTTCACATTCATATGGG
		MADS	ATGGGNMGNGGNAARATNGA	AGYTGYTGYTCNARYTGYTG

Results and Discussion

Cloning of flowering genes from Chinese cabbage

We have cloned 37 flowering-associated genes from Chinses Cabbage inbred lines, Chiifu (A line) and/or Kenshin (B line), by RT-PCR and submitted to GenBank after sequence analysis: Circadian rhythm related genes [*BrTOC1* (AY185117), *BrCCA1* (AY166504), *BrZTL* (AY185118)]; Photoperiod-realted genes [*BrPhyA* (AY324836), *BrPhyB* (AY176687), *BrCRY2* (AY176689), *BrFT* (AY176684), *BrCOP1* (AY166506), *BrDET1* (AY

324835), *BrHY5* (AY176679)]; Autonomous pathway genes [*BrFCA* (AY176672), *BrLD* (AY176680), *BrFRI* (AY176673), *BrFLC* (AY176676)]; Vernalization pathway gene [*BrVRN2* (AY 232729)]; Gibberellin signaling-associated genes [*BrGA4* (AY 176674), *BrGA5* (AY176675), *BrGAI* (AY176677), *BrSPY* (AY 188287), *BrRGAL* (AY185116)]; Flowering time related genes [*BrTFL* (AY176682), *BrLFY* (AY176685), *BrUFO* (AY232728), *BrCLF* (AY166505)]; Meristem identity-related genes [*BrAG* (AY162471), *BrAP2* (AY176683), *BrAP3* (AY232727), *BrPI* (AY 176688), *BrLUG* (AY176681), BrHUA2 (AY176678), *BrCRC* (AY220357), *BrNAP* (AY176686), *BrAGL20* (AY257541)]. However, we could not clone 7 genes from the Chinese cabbages; *LHY* (*LATE ELONGATED HYPOCOTYL*), *FKF1*, *GI*, *FWA*, *AP1* (*APETALA 1*), *CAL* (*CAULIFLOWER*) and *SPT* (*SPATULA*). However, it is not certain whether these genes are present in the Chinese cabbages or not. Therefore, we cloned these genes from *Arabidopsis thaliana* and used in this experiment. Most of the Chinese cabbage genes showed very high similarity to *Arabidopsis* counterparts at the level of nucleotide sequence (data not shown).

Effect of photoperiod on the expression of flowering genes

It is expected that the expression levels of flowering genes in Chinese cabbage will be changed by different photoperiod, LD or SD, and the levels will be different in two inbred lines, Chiifu and Kenshin. However, we obtained unexpected results in which expressions of many genes were similar in both lines as well as in both conditions, LD and SD (Figure 1). The expression of *LHY* gene was predominant in Chiifu line only under SD conditions. Genes, *TOC1* (*TIMING OF CAB 1*), *CR Y1* (*CRYPTOCHROME 1*), *CO*, *RGAL* (*REPRESSOR OF gal-LIKE*), *CLF*, *AGL20* and *AGL24*, were highly expressed in LD as well as SD conditions. The levels of transcripts for *CRY2* (*CRYPTOCHROME 2*), *GI*, and *GAI* (*GIBBERELLIC ACID INSENSITIVE*) were detectable, but not so for rest of the genes.

Mizoguchi et al. (2002) reported that *LHY and CCA1*, circadian rhythm-related genes, appeared to be negative regulatory elements required for central oscillator function. *GI* gene has also been shown to participate in the circadian system that is linked to overt rhythms in gene expression, leaf movements, hypocotyl elongation, and photoperiodic control of flowering in *Arabidopsis* (Sothern et al. 2002). We observed the expression of most circadian oscillator genes in both photoperiods. This warrants further study on the expression of *LHY* gene in the Chinese cabbage lines.

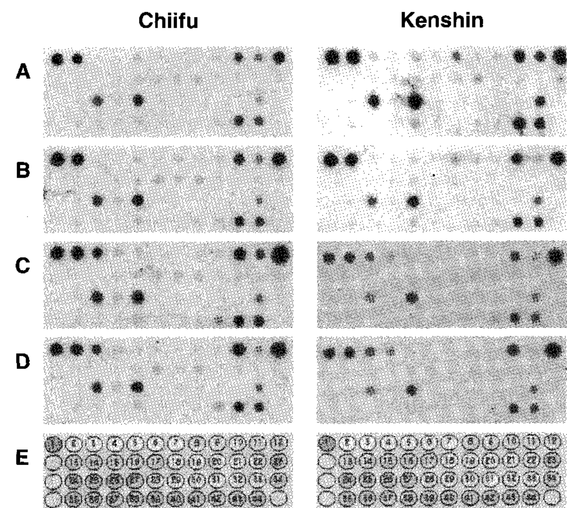

Figure 1. Expression of flowering genes in two Chinese cabbage lines under different photoperiods and light/dark conditions. Leaves were harvested at the end of each treatment and frozen in liquid nitrogen immediately. Probes were prepared from the total RNAs by reverse transcription. A: At the end of light period during a long day condition; B: At the end of dark period during a long day condition; C: At the end of light period during a short day condition; D: At the end of dark period during a short day condition; E: A position of each gene on the membrane: 1=β-*tubulin*, 2=*BrTOC1*, 3=*AtLHY*, 4=*BrCCA1*, 5=*AtFKF1*, 6=*BrZTL*, 7=*AtGI*, 8=*BrPhyA*, 9=*BrPhyB*, 10=*BrCRY1*, 11=*BrCRY2*, 12=*BrCO*, 13=*BrFT*, 14=*AtFWA*, 15=*BrCOP1*, 16=*BrDET1*, 17=*BrHY5*, 18=*BrFCA*, 19=*BrLD*, 20=*BrFRI*, 21=*BrFLC*, 22=*BrVRN2*, 23=*BrGA4*, 24=*BrGA5*, 25=*BrGAI*, 26=*BrSPY*, 27=*BrRGAL*, 28=*BrTFL*, 29=*BrLFY*, 30=*AtAP1*, 31=*AtCAL*, 32=*BrUFO*, 33=*BrCLF*, 34=*BrAG*, 35=*BrAP2*, 36=*BrAP3*, 37=*BrPI*, 38=*BrLUG*, 39=*BrHUA2*, 40=*BrCRC*, 41=*AtSPT*, 42=*BrNAP*, 43=*BrAGL20*, 44=*BrAGL24*.

Effect of vernalization on the expression of flowering genes

Generally Kenshin is sensitive to vernalization treatment while Chiifu is not. In Kenshin, treatment at 4°C for two weeks is enough to induce flowering. To find out which genes varied in expression during vernalization, we carried out dot blot analysis using the two inbred lines (Figure 2). Significant change in the mRNA levels was observed in two genes, *RGAL* and *NAP* (*NAC-LIKE*). The expression of *RGAL* gene was highly increased by vernalization in both inbred lines. However, the expression of *NAP* gene was induced by vernalization only in Chiifu inbred line. *RGA* appears to be a transcriptional regulator by controlling GA biosynthesis. *RGA* mRNA was expressed ubiquitously in all tissues (seedling, roots, rosette leaves, bolting stems, mature stems, flower buds, young siliqua, and mature siliqua) (Silverston et al. 1998). Therefore, the increase in *RGAL* transcript levels appears to be related to change in GA biosynthesis by vernalization.

The *NAP* expression was detected below the inflorescence meristem, young flower and ovules, and changed during devel-

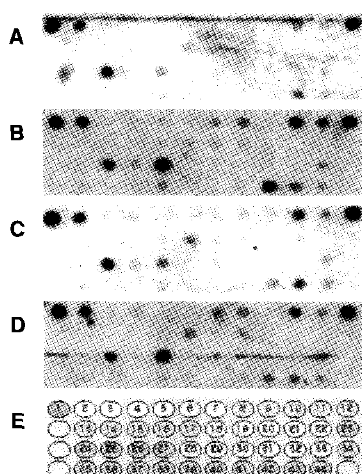

Figure 2. Effect of vernalization on expressions of flowering genes in Chinese cabbage. Seedlings subjected to 20 days vernalization were harvested and probes prepared from the freshly isolated RNAs by reverse transcription. A: Chiifu seedlings without vernalization; B: Chiifu seedlings after 20 days vernalization treatment; C: Kenshin seedlings without vernalization; D: Kenshin seedlings after 20 days vernalization; E: A position of each gene on the membrane: 1=*β-tubulin*, 2=*BrTOC1*, 3=*AtLHY*, 4=*BrCCA1*, 5=*AtFKF1*, 6=*BrZTL*, 7=*AtGI*, 8=*BrPhyA*, 9=*BrPhyB*, 10=*BrCRY1*, 11=*BrCRY2*, 12=*BrCO*, 13=*BrFT*, 14=*AtFWA*, 15=*BrCOP1*, 16=*BrDET1*, 17=*BrHY5*, 18=*BrFCA*, 19=*BrLD*, 20=*BrFRI*, 21=*BrFLC*, 22=*BrVRN2*, 23=*BrGA4*, 24=*BrGA5*, 25=*BrGAI*, 26=*BrSPY*, 27=*BrRGAL*, 28=*BrTFL*, 29=*BrLFY*, 30=*AtAP1*, 31=*AtCAL*, 32=*BrUFO*, 33=*BrCLF*, 34=*BrAG*, 35=*BrAP2*, 36=*BrAP3*, 37=*BrPI*, 38=*BrLUG*, 39=*BrHUA2*, 40=*BrCRC*, 41=*AtSPT*, 42=*BrNAP*, 43=*BrAGL20*, 44=*BrAGL24*.

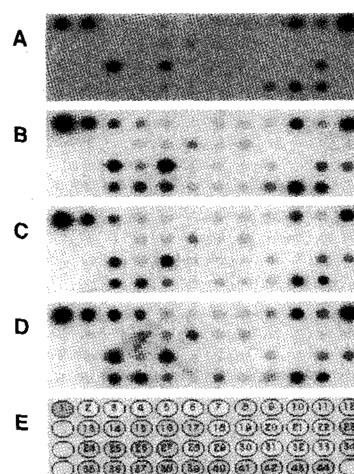

Figure 3. Transcript levels of flowering-associated genes during flower development of Chiifu line Chinese cabbage plants. A: Leaf samples; B: Early stage of flower bud formation; C: Fully mature floral buds; D: Flower opening stage; E: A position of each gene on the membrane: 1=*β-tubulin*, 2=*BrTOC1*, 3=*AtLHY*, 4=*BrCCA1*, 5=*AtFKF1*, 6=*BrZTL*, 7=*AtGI*, 8=*BrPhyA*, 9=*BrPhyB*, 10=*BrCRY1*, 11=*BrCRY2*, 12=*BrCO*, 13=*BrFT*, 14=*AtFWA*, 15=*BrCOP1*, 16=*BrDET1*, 17=*BrHY5*, 18=*BrFCA*, 19=*BrLD*, 20=*BrFRI*, 21=*BrFLC*, 22=*BrVRN2*, 23=*BrGA4*, 24=*BrGA5*, 25=*BrGAI*, 26=*BrSPY*, 27=*BrRGAL*, 28=*BrTFL*, 29=*BrLFY*, 30=*AtAP1*, 31=*AtCAL*, 32=*BrUFO*, 33=*BrCLF*, 34=*BrAG*, 35=*BrAP2*, 36=*BrAP3*, 37=*BrPI*, 38=*BrLUG*, 39=*BrHUA2*, 40=*BrCRC*, 41=*AtSPT*, 42=*BrNAP*, 43=*BrAGL20*, 44=*BrAGL24*.

opment of the above organs. Therefore, it was suggested that NAP functions in the transition between growth by cell division and cell expansion in stamens and petals (Sablowski and Meyerowitz 1998). It is unclear why *NAP* gene expression was induced in the seedlings of Chiifu A line by vernalization. We are trying to study the function of RGAL and NAP.

Expression of flowering genes during flower development

During flower development, flower identity-associated genes, such *AP1*, *AP2*, *PI* (*PISTILLATA*), *AGL20* and *AGL24*, were remarkably expressed when compared with leaf sample (Figure 3). Several other genes (*TOC*, *LHY*, *CRY1*, *CO*, *GA1* and *RGAL*) were also highly expressed. However, we could not detect the expression of eight genes, *FRI* (*FRIGDA*), *FLC*, *VRN2* (*VERNALIZATION 2*), *GA4*, *FT*, *AP1*, *TFL* (*TERMINAL FLOWER 1*), and *UFO* (*UNUSUAL FLORAL ORGANS*). These observations implied that the expression of flowering time-related genes can not be detectable in floral buds.

In conclusion, study on the expression of 43 flowering-related genes in two inbred lines of Chinese cabbage can be able to select three genes regulated by particular experimental conditions and to find out certain patterns of gene expression. The expression of *LHY* gene was predominant in Chiifu under the short-day conditions, whereas the expression of *RGAL* gene was increased by vernalization in both inbred lines. Besides, the expression of *NAP* gene was induced by vernalization only in Chiifu. Genes in two inbred lines, *TOC*, *CRY1*, *CO*, *RGAL* and *GAI*, were highly expressed under all tested conditions. However, the transcript level for several genes was not detected in this experiment.

Acknowledgments

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