Differential Expression of Flowering Genes between Rapid- and Slow-Cycling *Brassica rapa*

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ABSTRACT Flowering time is a very important agronomic trait in *Brassica* crops and regulation of the time is one of major factor in the breeding program. To understand the control of flowering time in *Brassica rapa*, we have carried out Br300K microarray with two contrasting *Brassica* inbred lines, Rapid Cycling *B. rapa* (RCBr) as rapid cycling type and *B. rapa* ssp. *pekinensis* inbred line Chiifu as slow flowering phenotype. Reproductive process-related genes were specifically expressed in RCBr, whereas environmental stimuli-responsive genes in Chiifu. Flowering stimulating genes, such as *BrFT* and *BrSOC1*, were preferentially expressed in RCBr, while flowering repressing genes, such as *BrFLC* and *BrMAF4*, expressed in Chiifu. Several paralogues present in *B. rapa*, *BrFLCs* and *BrCOLs*, were expressed with paralog-specific pattern depending on flowering phenotypes: i.e., *BrFLC1* and *BrFLC2*, major floral repressors, were expressed in Chiifu, *BrFLCL/BrFLC5* in RCBr and *BrFLC3* in both plants. The expression of several flowering repressing genes was gradually decreased in RCBr growth, but increased in Chiifu growth. However, the expression of genes involved in photoperiodic flowering was no difference between these two plants under LD and SD conditions, indicating photoperiodic pathway is not major factor to distinguish fast vs. slow flowering in *B. rapa*. The mechanism underlined in the rapid or fast flowering of RCBr would be further elucidated in association with the controlling mechanism of its short life span.

Keywords Br300K microarray, Flowering time, RCBr, Chiifu, Photoperiod

INTRODUCTION

Brassica rapa is an important crop species of the genus *Brassica*, which is cultivated worldwide and includes a variety of vegetable crops such as Chinese cabbage, Pak-choi, turnip, and turnip green, and oil seed crops including turnip rape and sarson (Gómez-Campo and Prakash 1999). *B. rapa* ssp. *pekinensis* (Chinese cabbage) belongs to one of nine subspecies, which is characterized by requirement of long period of cold treatment for flowering. Rapid cycling *B. rapa* (RCBr), also known as Fast Plant, is a widely used model organism in biology education (Williams and Hill 1986; Musgrave 2000). RCBr was developed by selection of *B. rapa* for several traits, such as short time to flowering, rapid seed maturation, lack of seed dormancy, petite growth habit, and high female fertility

(Williams and Hill 1986). The result is a plant with 10-generations per year. The earliness of flowering is an important trait for crops with higher seed yields (such as oilseed rape), whereas the lateness of flowering is a favourable trait for crops with high leafy head yields (such as Chinese cabbage) (Mao *et al.* 2014; Wang *et al.* 2014).

Flowering, a developmental process that transits vegetative stage to reproductive stage, is very important trait in *Brassica* species. Flowering in *Arabidopsis thaliana*, a model plant, is controlled by at least five genetically defined regulatory pathways: the photoperiod-dependent, vernalization-dependent, gibberellic acid (GA)-dependent, autonomous promotion, and FRIGIDA (FRI)-dependent pathways (Lee *et al.* 2000). Flowering time of the Chinese cabbage is largely controlled by vernalization and photoperiod (Kakizaki *et al.* 2011). Vernalization, exposure to

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low temperature, rerepresses FLOWERING LOCUS C (FLC) which is a central repressor of flowering (Helliwell *et al.* 2015). Plants with high FLC activity are late-flowering because FLC directly represses the expression of the floral inducers *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Helliwell *et al.* 2006). Four copies of *FLC* have been reported in *B. rapa*, and five copies were identified in *B. napus* (Lin *et al.* 2005; Kim *et al.* 2006; 2007). A genetic-genomics approach revealed that *BrFLC2* is a major regulator of flowering time in *B. rapa* (Xiao *et al.* 2013; 2014). Recently, Li *et al.* (2016) reported that mutation in *FLC1* and *FLC2* leading to production of truncated proteins was associated with shortness of flowering time in *B. rapa*. In photoperiodic flowering pathway, CONSTANS (CO) induces the day-length specific expression of *FT* in leaves (Putterill *et al.* 1995; Samach *et al.* 2000). Both CO and *FT* are mainly expressed in the vascular tissue of leaves (Takada and Goto 2003).

Although the regulation of flowering genes are important in *Brassica* breeding, information on RCB_r with respect to gene expression and rapid flowering is still unclear. To get insight on flowering-control mechanism, we have applied Br300K microarray (Dong *et al.* 2013) using two contrast *B. rapa* inbred lines, RCB_r as rapid cycling *B. rapa* and *B. rapa* ssp. *pekinensis* inbred line Chiifu as slow cycling *B. rapa*. Differentially expressed genes (DEGs) and major flowering genes were identified and characterized.

MATERIALS AND METHODS

Plant materials

Plant materials were rapid cycling *Brassica rapa* inbred line (RCBr24001) as an extremely early-flowering type and *B. rapa* ssp. *pekinensis*, inbred line Chiifu as slow or late flowering type. Plants were grown under either long day (LD; 16 hours light/8 hours dark) or short day (SD; 8 hours light/16 hours dark) conditions with $140 \pm 2 \mu\text{l/m}^2/\text{s}$ light intensity at $22^\circ\text{C} \pm 5^\circ\text{C}$. For microarray, shoots were sampled at both cotyledon stage and five-leaves stage of RCB_r (2 weeks old) and Chiifu (4 weeks old), and stored at

-70°C until use. For expression study during RCB_r development, RCB_r and Chiifu (as control) were sampled until RCB_r started flowering. To compare expression of several flowering genes, plants were grown under LD and SD conditions until 21 day after germination (DAG), and sampled at indicated time.

RNA isolation and hybridization to the Br300K microarray GeneChip

Shoot samples from cotyledon stage and five-leave stage were ground in liquid nitrogen, and equal amounts of powders were pooled. Total RNA was isolated from samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and further purified with a NucleoSpin RNA Clean-up Kit (Macherey-Nagel GmbH&Co. KG, Düren, Germany). For biological repeats, RNAs were extracted from two independent samples, and subjected to microarray experiment. Microarray experiments and subsequent analyses were performed as described in our previous report (Dong *et al.* 2013). To obtain insights regarding the putative biological functions and biochemical pathways of DEGs, we carried out enrichment analyses by searching Gene Ontology (GO) (Ashburner *et al.* 2000), agriGO (Du *et al.* 2010), and the Kyoto Encyclopedia of Genes and Genomes (Kanehisa *et al.* 2008).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), and the total RNA (1 μg) from each sample was subjected to the first-strand cDNA synthesis using the Ace- α kit with the Oligo(dT) primers (Toyobo, Osaka, Japan). Complementary DNA was diluted 10-fold and 1 μl of diluted cDNA was used in a 20- μl PCR mixture. RT-PCR primers were listed in Table 1, and primers for *BrACT2* (*B. rapa* *Actin 2*) used as control are 5'-GAA-CCGGGTGCTCCTCAGGA-3' (forward) and 5'-ATG-GTACCGGAATGGTCAAGGC-3' (reverse). To design paralog specific primer, expressed sequence tag (EST)-sequences were subjected to BRAD (<http://brassicadb.org/brad/>; V1.5), and all sequences were then aligned to figure out polymorphism. A standard PCR was performed with a 5-minute denaturation at 94°C , followed by 25

Table 1. List of primers used in qRT-PCR analysis.

Br_ID ²⁾	Gene	Gene annotation	Forward primer	Reverse primer
Bra004503	<i>BrCCA1</i>	CIRCADIAN CLOCK-ASSOCIATED 1	5'-CTTATGGACCTTCCGGTGAA	5'-TTCGAGCCACAAGAAGACCT
Bra008669	<i>BrCO</i>	CONSTANS	5'-GGCTCCTCAGGAAGTCACTACAA	5'-GCCATATCCTGTGTCATAGTACATTAGC
Bra008668	<i>BrCOL1-1</i>	CONSTANS LIKE-1-1	5'-GCAACAGACCAACTACCTGACC	5'-CGAAGGAACAACCTCATATCCTGTGTG
Bra023541	<i>BrCOL1-2</i>	CONSTANS LIKE-1-2	5'-CAACGGAGGAATTACCTGAAGCC	5'-TGAAGGAACAATTCCATATCCTGTGTCA
Bra001043	<i>BrCOL2-1</i>	CONSTANS LIKE-2-1	5'-CTGTTGTGCCAGAGTCAACAGAGAG	5'-TTTGCAAAACGTCCTTGATCCG
Bra021464	<i>BrCOL2-2</i>	CONSTANS LIKE-2-1	5'-CGTTAGTGTGTGCCAGAGTCATCAAG	5'-CTATCCTCTTTGCGAAACGGCCT
Bra032061	<i>BrCOL3</i>	CONSTANS LIKE-3	5'-ACTTTCCACACGGATTTCAG	5'-TCGCTTAGCAAAACGTCCTT
Bra040020	<i>BrCOL9-1</i>	CONSTANS LIKE-9-1	5'-GTTATGCAGCACATGCAAACTCACC	5'-GCTCTCAGCTGGTTGCACTGAG
Bra029666	<i>BrCOL9-2</i>	CONSTANS LIKE-9-2	5'-AGCCTTTAACACCTCTGGAGAGCTA	5'-ACCGGAGAGCTGCATTGAAGAC
Bra001264	<i>BrCOL9-3</i>	CONSTANS LIKE-9-3	5'-CAGTTCTGCAGCACAAGCAACAA	5'-GCTCCGTTTGAAGACTCATGAACGAGTC
Bra009055	<i>BrFLC1</i>	FLOWERING LOCUS C 1-1	5'-TCGTCACTCAAAAGCTTTGGACTGT	5'-TCGGAGATTGTCTCTGGTGAGACA
Bra028599	<i>BrFLC2</i>	FLOWERING LOCUS C 1-2	5'-CACACCATGAGCTACTAGAGCTTGTC	5'-GCAGTCGGAGAGTTACCGGAC
Bra006051	<i>BrFLC3</i>	FLOWERING LOCUS C 1-3	5'-CACACCATGAGCTACTAGAGCTTGTC	5'-GCAGTCGGAGAGTTACCGGAC
Bra001357	<i>BrFLD</i>	FLOWERING LOCUS D	5'-GGAGTGAAGCTTGTGGGAAG	5'-CACCGGTCCTTTAATCTGA
Bra023965	<i>BrKNAT5-1</i>	KNOTTED1-LIKE HOMEODOMAIN GENE 5-1	5'-GCCTGGAGACACAACGCTGTGTG	5'-TCACATAACCGAAGGAGAAACCATGTC
Bra011317	<i>BrKNAT5-2</i>	KNOTTED1-LIKE HOMEODOMAIN GENE 5-2	5'-AGAGCGGGAGAGATCCTTGC	5'-GCAAAACCGGTTTCTTCAACAGTTTC
Bra024351	<i>BrMAF4</i>	MADS AFFECTING FLOWERING 4	5'-CGGAAACTCTATASCTCTCC	5'-TTCCGATGACATTGCTCCTTCAC
Bra040518	<i>BrMBD9</i>	METHYL-CPG-BINDING DOMAIN 19	5'-CCTTATTCATTAGCAACTGAAGAAGGC	5'-AGCAGCCGCTGAAAGAGAAGAC
Bra006322	<i>BrSEP1-1</i>	SEPALLATA 1-1	5'-GCCAGCAAAAGGAATCTTCTTGGG	5'-GTGATGATGCTCGTACGGAACATT
Bra008674	<i>BrSEP1-2</i>	SEPALLATA 1-2	5'-CCTCTGAATTCAAAGGAGTTAGAGCAGA	5'-CACCAATCATATCATCCAGTTCATAGC
Bra021470	<i>BrSEP1-3</i>	SEPALLATA 1-3	5'-CTTGAGAGGACCTTGGACCC	5'-CTTCAATTGACAAAGCAGATTGGCG
Bra039170	<i>BrSEP1-4</i>	SEPALLATA 1-4	5'-GGGTCAACTAGACGGCTCTCTG	5'-GTGATGATGCTCGTACGGAACATT
Bra037544	<i>BrVRN1</i>	REDUCED VERNALIZATION RESPONSE 1	5'-CCGTTCAAAGTTCTACGAGAGTGC	5'-TCAGACGTACTCGTTGACTCGAWA
Bra021078	<i>BrVRN2</i>	REDUCED VERNALIZATION RESPONSE 2	5'-CAAGAAAGTTATCTGCTGAGCGATC	5'-TATTGCAGTTGTTGATGGTGGC

²⁾BRAD ID number identified by BlastN with expressed sequence tag sequence at <http://brassicadb.org/brad/>.

qRT-PCR: quantitative reverse transcription polymerase chain reaction.

cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds. PCR products were analyzed following electrophoresis through a 1.2% agarose gel.

RESULTS

Overall microarray

Among 47,553 EST clones deposited on Br300K microarray, 40,047 (84%) showed over 500 in probe intensity (PI), which means their transcripts could be detected by regular RT-PCR (Supplementary Table 1). In addition, 8,542 clones were not annotated with Arabidopsis gene locus (18% and expressed as 'no_hit_found', HNF). Estimated number of genes in *B. rapa* ssp. *pekinensis* inbred line Chiifu and RCBBr will be 44,710: 41,173 from BRAD (<http://brassicadb.org/brad/>; V1.5) (Wang *et al.* 2011), and 3,537 novel genes recently identified by deep RNA-Seq (Devisetty *et al.* 2014). Our microarray included more number of EST clones than the estimated numbers, but actual coverage of unigenes will be below 80% due to

overlapped EST clones.

Differentially or specifically expressed genes

Genes showing RCBBr-specific expression were defined as those genes with PI values greater than 1,000 in RCBBr but less than 500 in Chiifu (Supplementary Table 2). Chiifu-specific expression genes were selected with same strategy as RCBBr (Supplementary Table 3). RCBBr- and Chiifu-specifically expressed genes were 541 and 1,037, respectively. Among them, 178 (33%) and 446 (43%) genes from RCBBr and Chiifu, respectively, were NHF: i.e., no Arabidopsis counterpart to be present. These NHFs will be Brassica specific genes (or orphan genes). Only 363 and 591 genes from RCBBr and Chiifu, respectively, were annotated with Arabidopsis genome data. RCBBr-specific genes included many flowering-stimulating genes and transposable elements. On the other hand, Chiifu-specific genes included several floral repressor genes, such as *FLC* and *MAF4*.

To obtain insights regarding the putative biological functions and biochemical pathways of DEGs, we carried

out GO enrichment analyses by searching agriGO (Du *et al.* 2010) using RCB- (Supplementary Table 2) or Chiifu-induced genes (Supplementary Table 3). With the threshold value (P -value) below 0.05, 51 and 159 GO items were significantly enriched by the RCB- and Chiifu-induced genes, respectively (Fig. 1). GO items related reproductive processes were only significantly represented by RCB-induced genes, such as gametophyte development, flower development and cell differentiation. On the other hand, GO items related to stress response or environment stimulus were only significantly represented by Chiifu-induced genes. There are 27 GO Cellular component items were significantly represented in Chiifu-induced genes, and most of them related membrane structure, no such items were represented in RCB-induced genes.

Transcription factors (TFs)

Since TFs play roles as a master controller for plant growth and development, differentially expressed TF genes were analyzed (Table 2). In this analysis, we omitted TFs classified as flowering genes and then summarized as three categories; highly expressed-top 20 genes in both RCB and Chiifu, RCB-specific and Chiifu-specific genes. Ethylene-responsive TF RAP2-4 (*RAP2.4*) was the most highly expressed TF in both plants with over 40,000 in PI values. Genes expressed over two fold in RCB and Chiifu were 19 and 20, respectively; representative genes were *CAULIFLOWER (CAL)* and *SHINE2 (SHN2)* for RCB, and *KNOTTED1-LIKE HOMEODOMAIN GENE 5 (KNAT5)* and *CURLY LEAF (CLF)* for Chiifu.

Fig. 1. Functional categorization of differentially expressed genes between RCB and Chiifu based on gene ontology distribution.

Table 2. Transcription factors whose expressions levels were top 20 in both RCBBr and Chiifu, and over two-fold in RCBBr or Chiifu.

Classification	At_Locus	Gene annotation	Br_SEQ_ID	Probe intensity		Fold change	
				RCBr	Chiifu	R/C ²⁾	C/R
Top 20 in both RCBBr and Chiifu	AT1G78080	RAP2.4 (Ethylene-responsive transcription factor RAP2 4)	Brapa_ESTC021276	40264	40257	1.0	1.0
	AT3G54810	BME3/BME3-ZF (BLUE MICROPLYLAR END3)	Brapa_ESTC043727	38658	39227	1.0	1.0
	AT3G15210	ATERF4/ERF4 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 4)	Brapa_ESTC031149	38808	38636	1.0	1.0
	AT1G65440	GTB1 (GLOBAL transcription factor GROUP B1)	Brapa_ESTC034584	33003	38201	0.9	1.2
	AT5G59820	ZAT12 RHL41 (RESPONSIVE TO HIGH LIGHT 41)	Brapa_ESTC009473	34297	37970	0.9	1.1
	AT1G28370	ATERF11/ERF11 (ERF domain protein 11)	Brapa_ESTC031565	37200	36359	1.0	1.0
	AT1G20696	HMGB3 (HIGH MOBILITY GROUP B 3)	Brapa_ESTC008254	34477	35904	1.0	1.0
			Brapa_ESTC000857	35055	30406	1.2	0.9
	AT1G80840	WRKY40 (WRKY DNA-binding protein 40)	Brapa_ESTC014413	35089	33203	1.1	0.9
			Brapa_ESTC023350	33999	31364	1.1	0.9
	AT4G34410	AP2 domain-containing transcription factor, putative	Brapa_ESTC044996	30380	32899	0.9	1.1
	AT1G50600	SCL5 (Scarecrow-like protein 5)	Brapa_ESTC017171	30623	32226	1.0	1.1
	AT1G27730	ZAT10 STZ (SALT TOLERANCE ZINC FINGER)	Brapa_ESTC040097	42218	32094	1.3	0.8
	AT4G10710	SPT16 (GLOBAL transcription factor C)	Brapa_ESTC033093	29057	31334	0.9	1.1
	AT2G37630	MYB91 AS1 (ASYMMETRIC LEAVES 1)	Brapa_ESTC001152	31517	30950	1.0	1.0
	AT5G52660	MYB family transcription factor	Brapa_ESTC046861	38201	29175	1.3	0.8
	AT1G12610	DDF1 (DWARF AND DELAYED FLOWERING 1)	Brapa_ESTC039504	30466	29121	1.0	1.0
	AT5G48150	PAT1 (PHYTOCHROME A SIGNAL TRANSDUCTION 1)	Brapa_ESTC021790	38241	28474	1.3	0.7
	AT3G50060	MYB family transcription factor	Brapa_ESTC024817	38077	28352	1.3	0.7
	AT4G31550	WRKY11 (WRKY DNA-binding protein 11)	Brapa_ESTC044727	29815	28222	1.1	0.9
RCBr specific	AT5G21120	EIL2 (ETHYLENE-INSENSITIVE3-LIKE 2)	Brapa_ESTC037616	1460	116	12.6	.
	AT2G45680	TCP family transcription factor, putative	Brapa_ESTC042103	3852	377	10.2	.
	AT1G26310	CAL (CAULIFLOWER)	Brapa_ESTC017036	2570	287	9.0	.
	AT1G15050	IAA34 (indoleacetic acid-induced protein 34)	Brapa_ESTC033550	2023	250	8.1	.
	AT1G76890	GT2 (GT2)	Brapa_ESTC003989	1178	199	5.9	.
	AT5G25390	SHN2 (Ethylene-responsive transcription factor SHINE3)	Brapa_ESTC032163	1763	307	5.7	.
	AT1G73830	BEE3 (BR-ENHANCED EXPRESSION 3)	Brapa_ESTC019151	2796	496	5.6	.
	AT5G22290	ANAC089 (Arabidopsis NAC domain containing protein 89)	Brapa_ESTC021310	1579	281	5.6	.
			Brapa_ESTC034036	1104	301	3.7	.
	AT1G69560	MYB105 (myb domain protein 105)	Brapa_ESTC040454	1409	277	5.1	.
	AT5G15310	AtMYB16 (myb domain protein 16)	Brapa_ESTC025442	1817	419	4.3	.
	AT1G20530	Unknown (similar to bzip-related transcription factor -like)	Brapa_ESTC000221	1921	497	3.9	.
	AT5G60890	AtMYB34 ATR1 (ALTERED TRYPTOPHAN REGULATION)	Brapa_ESTC026773	1226	326	3.8	.
	AT1G17590	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	Brapa_ESTC003250	1089	302	3.6	.
	AT4G33280	AP2/B3-like transcription factor	Brapa_ESTC044866	1607	480	3.3	.
	AT2G03060	Similar to AGL102	Brapa_ESTC019197	1325	401	3.3	.
	AT3G12720	AtMYB67/AtY53 (myb domain protein 67)	Brapa_ESTC042873	1358	467	2.9	.
	AT5G06839	bZIP family transcription factor	Brapa_ESTC045391	1162	455	2.6	.
	AT4G32280	IAA29 (indoleacetic acid-induced protein 29)	Brapa_ESTC035154	1126	472	2.4	.
	AT1G15360	SHN1/WIN1 (SHINE1/WAX INDUCER 1)	Brapa_ESTC017040	1064	464	2.3	.
Chiifu specific	AT4G32040	KNAT5 (KNOTTED1-LIKE HOMEBOX GENE 5)	Brapa_ESTC025634	44	18085	.	413.2
	AT5G51190	AP2 domain-containing transcription factor, putative	Brapa_ESTC013139	145	12449	.	85.7
	AT4G01550	ANAC069 (Arabidopsis NAC domain containing protein 69)	Brapa_ESTC007789	95	7576	.	80.0
	AT3G50260	CEJ1 (COOPERATIVELY REGULATED BY ETHYLENE AND JASMONATE 1)	Brapa_ESTC001959	17	1102	.	66.0
	AT5G60890	AtMYB34 ATR1 (ALTERED TRYPTOPHAN REGULATION)	Brapa_ESTC009043	408	22870	.	56.1
	AT2G22430	ATHB6 (ARABIDOPSIS THALIANA HOMEBOX PROTEIN 6)	Brapa_ESTC004579	323	13848	.	42.9
	AT2G46970	PIL1 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 1)	Brapa_ESTC042137	108	3570	.	33.1
	AT2G42780	similar to Os08g0169600	Brapa_ESTC050862	49	1577	.	32.1
	AT1G49950	ATTRB1/TRB1 (TELOMERE REPEAT BINDING FACTOR 1)	Brapa_ESTC051029	270	4988	.	18.5
	AT4G12020	MAPKKK11 WRKY19 (WRKY DNA-binding protein 19)	Brapa_ESTC030987	149	2540	.	17.1
			Brapa_ESTC037364	397	5334	.	13.4
	AT5G59820	ZAT12 RHL41 (RESPONSIVE TO HIGH LIGHT 41)	Brapa_ESTC006145	106	1379	.	13.0
	AT4G31800	WRKY18 (WRKY DNA-binding protein 18)	Brapa_ESTC001172	320	3971	.	12.4
	AT3G27810	ATMYB21 (MYB DOMAIN PROTEIN 21)	Brapa_ESTC008010	445	4910	.	11.0
	AT3G61150	HDG1 (HOMEODOMAIN GLABROUS1)	Brapa_ESTC022251	201	1813	.	9.0
	AT3G28920	ATHB34 (ARABIDOPSIS THALIANA HOMEBOX PROTEIN 34)	Brapa_ESTC051903	418	3716	.	8.9
	AT3G11100	Transcription factor	Brapa_ESTC005968	361	2986	.	8.3
	AT2G23380	CLF (CURLY LEAF)	Brapa_ESTC048306	138	1075	.	7.8
	AT1G71030	ATMYBL2 (Arabidopsis myb-like 2)	Brapa_ESTC021435	249	1760	.	7.1
	AT5G16820	HSFA1B HSF3 (HEAT SHOCK FACTOR 3)	Brapa_ESTC036030	481	1465	.	3.0
	AT5G61590	AP2 domain-containing transcription factor family protein	Brapa_ESTC021781	465	1249	.	2.7

²⁾R/C and C/R indicate the expression ratio in RCBBr over Chiifu and Chiifu over RCBBr, respectively. Flowering-related TF genes were omitted in this Table.

Flowering genes

Genes listed in Table 3 were selected from 196 flowering-related genes on Br300K microarray: 26, 9 and 23 genes for up-regulated in RCB, in Chiifu and both, respectively. Genes (26 genes) up-regulated in RCB included most flowering-promoting genes, such as *FT* and

SOC1. On the other hand, genes (9) up-regulated in Chiifu included floral repressors, such as *FLC* and *MAF4*. Twenty-three genes were highly expressed in both RCB and Chiifu, which included *DWARF AND DELAYED FLOWERING 1 (DDF1)* and *EARLY FLOWERING 3 (ELF3)*.

Interestingly, expression of several flowering genes

Table 3. Flowering-related genes which were selected from 196 flowering genes on Br300K microarray.

Classification	At_Locus	Gene annotation	Br_SEQ_ID	Probe intensity		Fold change	
				RCBr	Chiifu	R/C ²⁾	C/R
Up-regulated genes in RCB	AT5G20240	PI (PISTILLATA)	Brapa_ESTC028268	4123	43	95.0	.
			Brapa_ESTC008132	6436	365	17.7	.
			Brapa_ESTC034308	4875	799	6.1	.
			Brapa_ESTC048371	872	163	5.3	.
			Brapa_ESTC019275	3390	359	9.4	.
	AT5G15800	SEP1 (SEPALLATA1)/AGL2	Brapa_ESTC048372	3473	963	3.6	.
			Brapa_ESTC025908	1629	32	50.6	.
			Brapa_ESTC011597	2267	94	24.2	.
			Brapa_ESTC045715	7932	402	19.7	.
			Brapa_ESTC040402	8412	267	31.5	.
	AT1G69120	AP1 (APETALA1)/AGL4	Brapa_ESTC017264	4988	242	20.6	.
			Brapa_ESTC010543	527	26	20.2	.
			Brapa_ESTC008651	5851	313	18.7	.
			Brapa_ESTC008039	5018	200	25.0	.
			Brapa_ESTC005886	1654	69	24.0	.
	AT3G02310	SEP2 (SEPALLATA2)	Brapa_ESTC031079	4813	254	19.0	.
			Brapa_ESTC049045	1242	197	6.3	.
			Brapa_ESTC010485	2902	138	21.0	.
			Brapa_ESTC007881	6834	1754	3.9	.
			Brapa_ESTC008198	1927	93	20.6	.
	AT4G18960	AG (AGAMOUS)	Brapa_ESTC018123	2371	253	9.4	.
			Brapa_ESTC010831	8934	615	14.5	.
			Brapa_ESTC018131	2547	932	2.7	.
			Brapa_ESTC010942	1086	112	9.7	.
			Brapa_ESTC017036	2570	287	9.0	.
	AT1G24260	SEP3 (SEPALLATA3)/AGL9	Brapa_ESTC034579	1985	278	7.1	.
			Brapa_ESTC019941	2432	462	5.3	.
			Brapa_ESTC027877	5149	819	6.3	.
			Brapa_ESTC051650	3495	1501	2.3	.
			Brapa_ESTC011095	14204	3600	3.9	.
	AT2G45650	AGL6 (AGAMOUS LIKE-6)	Brapa_ESTC018989	10579	3022	3.5	.
			Brapa_ESTC019044	9961	3960	2.5	.
			Brapa_ESTC017129	22280	5691	3.9	.
			Brapa_ESTC018052	4399	1195	3.7	.
			Brapa_ESTC029975	2502	1078	2.3	.
	AT1G26310	CAL (CAULIFLOWER)/AGL10	Brapa_ESTC008221	3953	1108	3.6	.
			Brapa_ESTC045284	898	253	3.5	.
			Brapa_ESTC019197	1325	401	3.3	.
			Brapa_ESTC043198	4038	1494	2.7	.
			Brapa_ESTC023599	3673	1782	2.1	.
	AT1G65480	FT (FLOWERING LOCUS T)	Brapa_ESTC012017	749	288	2.6	.
			Brapa_ESTC018786	4340	1879	2.3	.
			Brapa_ESTC034818	9026	3696	2.4	.
			Brapa_ESTC020072	2427	1002	2.4	.
			Brapa_ESTC019071	5260	2182	2.4	.
	AT3G07650	COL9 (CONSTANS-LIKE 9)	Brapa_ESTC033456	1381	590	2.3	.
			Brapa_ESTC027067	1108	485	2.3	.
			Brapa_ESTC017216	8885	4014	2.2	.
			Brapa_ESTC010865	5636	2491	2.3	.
			Brapa_ESTC041607	1624	809	2.0	.
	AT2G45660	SOC1/AGL20 (AGAMOUS-LIKE 20)(SOC1-2, SOC1-1, SOC1-3)					
	AT5G60910	FUL/AGL8 (AGAMOUS-LIKE 8)					
	AT5G24860	FPF1 (FLOWERING PROMOTING FACTOR 1)					
	AT5G62165	AGL42 (AGAMOUS LIKE 42)					
	AT5G03840	TFL1 (TERMINAL FLOWER 1)					
	AT2G03060	Similar to AGL102					
	AT3G21320	Similar to ELF3 (EARLY FLOWERING 3)					
	AT2G43010	PIF4 (PHYTOCHROME INTERACTING FACTOR 4)					
	AT2G45830	DTA2 (DOWNSTREAM TARGET OF AGL15 2)					
	AT5G15850	COL1 (CONSTANS-LIKE 1)					
	AT3G59060	PIF5/ PIL6 (PHYTOCHROME-INTERACTING FACTOR 5)					
	AT1G71692	AGL12 (AGAMOUS-LIKE 12)					
	AT5G10140	FLC (FLOWERING LOCUS C)/AGL25(FLCL/FLC5)					
	AT5G13790	AGL15 (AGAMOUS-LIKE 15)					
	AT2G33350	Similar to zinc finger CONSTANS-related					

²⁾R/C and C/R indicate the expression ratio in RCB over Chiifu and Chiifu over RCB, respectively.

Table 3. Continued.

Classification	At_Locus	Gene annotation	Br_SEQ_ID	Probe intensity		Fold change	
				RCBr	Chiifu	R/C ⁽²⁾	C/R
Up-regulated genes in Chiifu	AT5G10140	FLC (FLOWERING LOCUS C)/AGL25(FLC1, FLC2)	Brapa_ESTC016533	53	1504	.	28.3
			Brapa_ESTC012276	6738	13305	.	2.0
	AT5G65070	FCL4/MAF4 (MADS AFFECTING FLOWERING 4)	Brapa_ESTC013135	393	8499	.	21.6
			Brapa_ESTC046597	986	4411	.	4.5
			Brapa_ESTC013362	3209	11579	.	3.6
			Brapa_ESTC046600	300	1051	.	3.5
			Brapa_ESTC046598	3151	9784	.	3.1
	AT1G31140	AGL63 (AGAMOUS-LIKE 63)	Brapa_ESTC040293	206	2363	.	11.5
	AT5G04240	ELF6 (EARLY FLOWERING 6)	Brapa_ESTC020902	368	1517	.	4.1
	AT5G13790	AGL15 (AGAMOUS-LIKE 15)	Brapa_ESTC020193	1290	4174	.	3.2
	AT1G30260	AGL79 (AGAMOUS-LIKE 79)	Brapa_ESTC014642	276	776	.	2.8
	AT1G77080	AGL27/MAF1 (MADS AFFECTING FLOWERING 1)	Brapa_ESTC022419	1327	3363	.	2.5
	AT1G09530	PIF3 (PHYTOCHROME INTERACTING FACTOR 3)	Brapa_ESTC026673	2332	5556	.	2.4
	AT3G57390	AGL18 (AGAMOUS-LIKE 18)	Brapa_ESTC021222	2876	5626	.	2.0
Highly expressed genes in both RCB and Chiifu	AT1G12610	DDF1 (DWARF AND DELAYED FLOWERING 1)	Brapa_ESTC039504	30466	29121	1.0	1.0
			Brapa_ESTC002858	19586	15570	1.3	0.8
	AT2G25930	ELF3 (EARLY FLOWERING 3)	Brapa_ESTC041284	26959	22606	1.2	0.8
			Brapa_ESTC041298	22936	16177	1.4	0.7
			Brapa_ESTC015252	19828	20034	1.0	1.0
	AT1G25540	PFT1 (PHYTOCHROME AND FLOWERING TIME 1)	Brapa_ESTC047777	23968	21573	1.1	0.9
	AT2G22540	AGL22/SVP (SHORT VEGETATIVE PHASE)	Brapa_ESTC014320	23538	18005	1.3	0.8
			Brapa_ESTC050176	8869	9304	1.0	1.0
			Brapa_ESTC041116	18874	17799	1.1	0.9
	AT3G04610	FLK (FLOWERING LOCUS KH DOMAIN)	Brapa_ESTC042608	21959	17640	1.2	0.8
	AT5G62640.2	ELF5 (EARLY FLOWERING 5)	Brapa_ESTC001982	19200	13055	1.5	0.7
	AT2G24790.1	COL3 (CONSTANS-LIKE 3)	Brapa_ESTC011117	19001	27161	0.7	1.4
			Brapa_ESTC014037	15458	24927	0.6	1.6
			Brapa_ESTC027269	13996	19872	0.7	1.4
	AT5G10140	FLC (FLOWERING LOCUS C)/AGL25 (FLC3)	Brapa_ESTC013303	17486	16533	1.1	0.9
			Brapa_ESTC021830	13579	14983	0.9	1.1
	AT4G24540	AGL24 (AGAMOUS-LIKE 24)	Brapa_ESTC036920	17200	17618	1.0	1.0
			Brapa_ESTC019065	14515	13686	1.1	0.9
	AT1G77300	EFS (EARLY FLOWERING IN SHORT DAYS)	Brapa_ESTC040684	17197	13552	1.3	0.8
	AT4G22950	AGL19 (AGAMOUS-LIKE 19)	Brapa_ESTC044364	16772	15569	1.1	0.9
			Brapa_ESTC018998	12476	12321	1.0	1.0
	AT3G12810	PIE1 (PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1)	Brapa_ESTC042862	16091	12137	1.3	0.8
			Brapa_ESTC024641	14327	8484	1.7	0.6
	AT2G06210	ELF8 (EARLY FLOWERING 8)	Brapa_ESTC004485	15388	12464	1.2	0.8
			Brapa_ESTC042284	12694	10565	1.2	0.8
	AT1G07050	CONSTANS-like protein-related	Brapa_ESTC021115	15190	10154	1.5	0.7
	AT1G77080	AGL27, MAF1 (MADS AFFECTING FLOWERING 1)	Brapa_ESTC000396	14927	10695	1.4	0.7
	AT3G07650	COL9 (CONSTANS-LIKE 9)	Brapa_ESTC042798	14472	8979	1.6	0.6
			Brapa_ESTC051649	12413	8766	1.4	0.7
			Brapa_ESTC021535	12256	7067	1.7	0.6
			Brapa_ESTC025316	8692	4979	1.7	0.6
			Brapa_ESTC049114	7570	5204	1.5	0.7
			Brapa_ESTC049113	7031	5176	1.4	0.7
			Brapa_ESTC006144	6478	9542	0.7	1.5
	AT4G28190	ULT1 (ULTRAPETALA1)	Brapa_ESTC044575	14100	11058	1.3	0.8
	AT1G79730	ELF7 (EARLY FLOWERING 7)	Brapa_ESTC006396	12278	10830	1.1	0.9
			Brapa_ESTC009946	8400	10613	0.8	1.3
	AT4G36920	AP2 (APETALA 2)	Brapa_ESTC034160	10951	9936	1.1	0.9
			Brapa_ESTC007967	7301	8061	0.9	1.1
			Brapa_ESTC012323	4120	5899	0.7	1.4
			Brapa_ESTC013840	3821	4959	0.8	1.3
			Brapa_ESTC023600	2272	1607	1.4	0.7
	AT3G04610	FLK (FLOWERING LOCUS KH DOMAIN)	Brapa_ESTC021699	10239	6514	1.6	0.6
			Brapa_ESTC015151	8990	9444	1.0	1.1
	AT2G45660	SOC1/AGL20 (AGAMOUS-LIKE 20)(SOC1-3)	Brapa_ESTC034801	9787	7499	1.3	0.8
	AT2G40080	ELF4 (EARLY FLOWERING 4)	Brapa_ESTC038745	9602	6902	1.4	0.7
			Brapa_ESTC023094	8429	13088	0.6	1.6
			Brapa_ESTC027935	6642	4190	1.6	0.6
	AT5G15850	COL1 (CONSTANS-LIKE 1)	Brapa_ESTC013309	9416	16434	0.6	1.7

showed paralog-specific pattern: i.e., *BrFLC1* (Bra009055; Brapa_ESTC016533) and *BrFLC2* (Bra028599; Brapa_ESTC012276) in Chiifu, *BrFLC1/BrFLC5* (FLC-Like, Bra022771; Brapa_ESTC017216 and Brapa_ESTC027067) in RCB, and *BrFLC3* (Bra006051; Brapa_ESTC013303 and Brapa_ESTC021830) in both plants. *CONSTNAS-LIKE 9 (COL9)* was also paralog specific expression: Brapa_ESTC027877 (Bra029666; *BrCOL9-2*) and Brapa_ESTC051650 (Bra001264; *BrCOL9-3*) for up-regulated ones in RCB, but rest (most of them are *BrCOL9-1*; Bra040020) in both plants. These paralog-specific expressions of flowering genes were highlight of this study.

Gibberellin metabolism-related genes

Since gibberellins (GAs) control flowering in concert with cytokinin (Matías-Hernández *et al.* 2016), the expression of GA-metabolism-related genes were examined (Table 4). Most genes involved in increase of the GA activity were up-regulated in RCB; *GA3OX1/GA4* (GA requiring 4) and *GA20OX3* (Gibberellin 20 oxidase 3). Only *GA2OX2* (Gibberellin 2-beta-dioxygenase) expression

was high in Chiifu, but its function has not been identified besides its involvement in GA catabolism.

Confirmation of selected gene expression

Fig. 2 showed genes whose expression was decreased during the development of RCB, while increased in Chiifu. These included *MADS AFFECTING FLOWERING 4 (FCL4/MAF4)*, *FLC2*, *FLOWERING LOCUS D (FLD)*, *REDUCED VERNALIZATION RESPONSE 1 (VRN1)*, *REDUCED VERNALIZATION RESPONSE 2 (VRN2)*, Polycomb group), *METHYL-CPG-BINDING DOMAIN 9 (MBD9)* and *KNAT5*. Most of them are known to be floral repressors. The expression of these genes was gradually increased in Chiifu even though this period belongs to be very early developmental stages.

Photoperiod is important for controlling flowering of *Brassica* species. To elucidate its association, several TF genes and flowering-related genes were selected and their expressions were examined during a day under LD and SD conditions. Plants grown for 21 DAG under LD and SD were sampled at indicated time (Fig. 3A). *B. rapa*

Table 4. Genes related to gibberellic acid metabolism.

At_Locus	Gene annotation	Br_SEQ_ID	Prove intensity		Fold change	
			RCBr	Chiifu	R/C ²⁾	C/R
AT1G30040	GA2OX2 (Gibberellin 2-beta-dioxygenase)	Brapa_ESTC040202	1319	6104	0.2	4.6
		Brapa_ESTC015031	16	1969	0.0	126.4
AT1G02400	GA2OX6/DTA1 (Gibberellin 2-OXIDASE 6)	Brapa_ESTC015242	10762	8386	1.3	0.8
AT4G21200	GA2OX8 (Gibberellin 2-OXIDASE 8)	Brapa_ESTC024684	431	665	0.6	1.5
		Brapa_ESTC015062	423	624	0.7	1.5
AT1G15550	GA3OX1/GA4 (GA REQUIRING 4)	Brapa_ESTC027719	554	164	3.4	0.3
AT5G07200	GA20OX3/YAP169 (Gibberellin 20 oxidase 3)	Brapa_ESTC045377	1296	155	8.4	0.1
		Brapa_ESTC029845	4761	183	26.1	0.0
		Brapa_ESTC027440	1114	81	13.7	0.1
		Brapa_ESTC003065	9782	9954	1.0	1.0
AT5G14920	Gibberellin-regulated family protein	Brapa_ESTC016390	5777	9747	0.6	1.7
		Brapa_ESTC042364	10773	8665	1.2	0.8
		Brapa_ESTC024407	6879	6685	1.0	1.0
		Brapa_ESTC007229	5209	4283	1.2	0.8
AT5G59845	Gibberellin-regulated family protein	Brapa_ESTC010071	5161	3227	1.6	0.6
		Brapa_ESTC007822	507	20	25.9	0.0
AT1G74670	Gibberellin-responsive protein, putative	Brapa_ESTC037994	4417	3177	1.4	0.7
		Brapa_ESTC008005	3581	2190	1.6	0.6
AT1G22690	Gibberellin-responsive protein, putative	Brapa_ESTC003796	5081	1538	3.3	0.3
AT5G51310	Gibberellin 20-oxidase-related	Brapa_ESTC046793	859	1135	0.8	1.3
AT3G10185	Gibberellin-regulated GASA/GAST/Snakin family protein	Brapa_ESTC036747	4237	5060	0.8	1.2
AT4G26420	GAMT1 Gibberellin carboxyl-O-methyltransferase	Brapa_ESTC019836	1067	287	3.7	0.3
AT5G56300	GAMT2 Gibberellin carboxyl-O-methyltransferase	Brapa_ESTC009976	773	115	6.7	0.1

²⁾R/C and C/R indicate the expression ratio in RCB over Chiifu and Chiifu over RCB, respectively.

Fig. 2. Genes whose expression was decreased in RCB, while increased in Chiifu. RCB and Chiifu were grown in long-day condition for 19 days and shoot was sampled at 4th hour of light period.

Fig. 3. Expression of flowering- and photoperiod pathway-related genes. (A) Photoperiod for plant growth and sampling time a day. (B) Genes showing differential expression in a microarray. (C) *Constance* (*CO*) and its paralogs. *BrCCA1* was used as control for circadian rhythm. LD: long-day condition, SD: short-day condition.

CIRCADIAN CLOCK ASSOCIATED 1 (*BrCCA1*) was used as a control for the circadian clock. The expression of *BrFLC2* was high in Chiifu, but not detected in RCB. Individual paralogues of *BrSEPI* (*SEPALLATA1/AGL2*) were specific for either plant (Fig. 3B). However, photoperiodic pathway integrator, *CO* and its relatives (*COLs*) were expressed with similar patterns in both plants

(Fig. 3C). Only *BrCOL2-1* was expressed in Chiifu. Taken together, there was no photoperiodic-dependent expression, but the expression of *BrFLC2*, *BrSEPIs* and *BrCOL2-1* was plant-specific. These daily expression patterns was not agreement with microarray data, due to sampling time difference.

DISCUSSION

Because of striking difference in periods required for flowering between RCBBr and *B. rapa* ssp. *pekinensis*, inbred line Chiifu, normalization of developmental stages of these two plants would be almost impossible. Therefore, identification of master gene(s) for controlling flowering time would be also very difficult. However, microarray data in this study showed remarkable difference in the expression of flowering-related genes between two plants; high expression of flowering promoting genes in RCBBr, whereas high expression of floral repressor genes in Chiifu. DEGs between RCBBr and Chiifu included many NHFs with respect to BlastN against *Arabidopsis* gene ID, suggesting that these would be *Brassica* species-specific genes. That can be supported by that most sequences were matched to the public genome data or NCBI data for *B. napus* and *B. oleracea*. For example, Brapa_ESTC051261 was the most highly expressed gene (937 fold) in Chiifu compared with RCBBr. As result of BlastN in RCBBr, this clone was matched to 100% with *B. napus* uncharacterized LOC106444620, ncRNA and 90% with *B. oleracea* var. *oleracea* uncharacterized LOC106327354. Brapa_ESTC033111 was the most highly expressed gene (301 fold) in RCBBr: 93% identical to *B. napus* uncharacterized LOC106448050 and 92% to *B. oleracea* var. *oleracea* uncharacterized LOC106330763. This finding strongly suggests that functional study on these *Brassica*-specific genes will be very important for understanding evolution of Brassicaceae crops.

Analysis of DEGs

GO enrichment analysis with DEGs showed distinct difference between RCBBr, very early flowering phenotype, and Chiifu, a slow or late flowering phenotype (Fig. 1). Flowering process-related genes were specifically induced in RCBBr, whereas temperature and other environment stress tolerant-related genes were expressed in Chiifu. Some general biological processes were conserved in both RCBBr and Chiifu. This result might imply that Chiifu is not ready to flower yet. However, it was ruled out whether this difference is due to the difference in developmental stages during whole life cycle.

Expression of TF genes

TFs control the expression of a large number genes, thereby exerting big impact on plant morphogenesis. As shown in Table 2, many TFs were highly expressed in both plants, some were specifically expressed in RCBBr or in Chiifu. Based on current knowledge, it was hard to find out the specific association with growth and development for highly expressed TFs in both plants; i.e., *RAP2.4*, the most highly expressed gene, is known to promote leaf senescence (Xu *et al.* 2010). However, several genes among RCBBr-induced TFs seemed to be closely related to early flowering phenotype. TCP family determines in the height of inflorescence shoot (Davière *et al.* 2014), which might be related to short height of RCBBr. SHN2 is known to be related to flower organ (Shi *et al.* 2011), and *CAULIFLOWER* (*CAL*) is closely related to another MADS box gene *APETALA1* (*AP1*) and control floral meristem development (Alvarez-Buylla *et al.* 2000).

Regarding to genes induced in Chiifu, most TFs appeared to be related to plant growth and development as follows. KNAT5 regulates photomorphogenic responses and represses late steps in gibberellin biosynthesis. (Hay *et al.* 2002). AP2 encoding a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 TF family may be related to the ethylene-singalling pathway. AtMYB34 regulates indole glucosinolate homeostasis (Celenza *et al.* 2005). ATHB6, a target of the protein phosphatase ABI1, regulates negatively ABA responses in *Arabidopsis* (Himmelbach *et al.* 2002). Only CLF, a repressor of AG function (Goodrich *et al.* 1997), is related to flowering; it suppresses flowering by inducing misexpression of floral promoting genes (Lopez-Vernaza *et al.* 2012)

Expression of GA metabolism-related genes

GA content is associated with fertility (Plackett *et al.* 2012). GA 20-oxidase (GA20ox) regulates GA content (Yamaguchi 2008), and there are five GA20ox genes in *Arabidopsis*, *GA20ox1*, *GA20ox2*, *GA20ox3*, *GA20ox4*, and *GA20ox5* (Hedden *et al.* 2002). Among them, *GA20ox3* functions almost entirely redundantly with *GA20ox1* and *GA20ox2* at most developmental stages, including the floral transition, thereby these genes control

floral organ growth and anther development (Plackett *et al.* 2012). In our results (Table 4), *GA20OX3* was highly expressed in RCB, suggesting the possible function in flowering. In addition, several GA responsive genes were up-regulated in RCB, but their role in flowering have to be solved. Only one gene, *GA20OX2* (Gibberellin 2-beta-dioxygenase) was up-regulated in Chiifu, but the function of this gene has not been identified yet.

Expression of flowering-related genes

Regarding to flowering-related genes, several flowering-promoting genes, such as *PISTILLATA (PI)*, *SEP1* and *API*, were up-regulated in RCB, whereas floral repressors, such *FLC* and *MAF4*, were up-regulated in Chiifu (Table 3). These results can partially support why these two plants exhibit contrasting flowering phenotypes, rapid vs. slow. Interestingly, several genes contain paralogues; *FLC*, *COL9* and *SOC1*. Schranz *et al.* (2002) and Franks *et al.* (2015) reported presence of four *FLC* paralogues in *B. rapa* (*BrFLC1*, *BrFLC2*, *BrFLC3*, and *BrFLC5*). A genetic-genomics approach revealed that *BrFLC2* is a major regulator of flowering time in *B. rapa* (Wu *et al.* 2012; Xiao *et al.* 2013; 2014). Recently, Li *et al.* (2016) reported that early flowering phenotypes of *B. rapa* is related to be nonfunctional *BrFLC*s which are truncated form by premature termination, but slow or late flowering types contain functional or intact forms of *BrFLC1*, *BrFLC2*, and *BrFLC3*. In addition, Song *et al.* (2015) found out that expression of only one or some of paralogues is closely related to photoperiodic flowering in *B. rapa*. All these facts might be similar to our study: i.e., *BrFLC2* were up-regulated in Chiifu, a slow or late flowering phenotype. However, *BrFLC5* was up-regulated in RCB, suggesting the another possible role of *FLC* besides flowering control.

RT-PCR results of selected genes demonstrated that the expression of flowering repressor genes was gradually decreased in RCB, while gradually increased in Chiifu (Fig. 2). These included *BrMBD9*, *BrVRN1*, *BrVRN2*, *BrFLC*, and *BrFLD*. *MBD9* controls flowering time by modulating gene expression through DNA methylation and histone acetylation (Yaish *et al.* 2009). *VRN1* and *VRN2* affect vernalization response of late flowering plants (Chandler *et al.* 1996). *FLD* (*Flowering Locus D*)

encodes a plant ortholog of the human Lys-Specific Demethylase 1 (LSD1) protein. *FLD* functions in histone H3K4 demethylation and H3/H4 deacetylation to repress the expression of *FLC* (Jiang *et al.* 2007; Yu *et al.* 2011). Our data well agree with previous findings.

It was expected that photoperiodic flowering signaling-related genes are differentially expressed between RCB and Chiifu, but no significant difference was observed (Fig. 3). Special attention paid to *COL9* (*CONSTANS-LIKE 9*) which delays flowering by down-regulation of expression of *CO*, *FT*, and *SOC1* (Cheng and Wang 2005). However, three paralogues showed similar expression patterns between two plants.

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