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RESEARCH PAPER

BrFLC2 (*FLOWERING LOCUS C*) as a candidate gene for a vernalization response QTL in *Brassica rapa*

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Abstract

Flowering time is an important agronomic trait, and wide variation exists among *Brassica rapa*. In *Arabidopsis*, *FLOWERING LOCUS C* (*FLC*) plays an important role in modulating flowering time and the response to vernalization. *Brassica rapa* contains several paralogues of *FLC* at syntenic regions. *BrFLC2* maps under a major flowering time and vernalization response quantitative trait locus (QTL) at the top of A02. Here the effects of vernalization on flowering time in a double haploid (DH) population and on *BrFLC2* expression in selected lines of a DH population in *B. rapa* are descibed. The effect of the major flowering time QTL on the top of A02 where *BrFLC2* maps clearly decreases upon vernalization, which points to a role for *BrFLC2* underlying the QTL. In all developmental stages and tissues (seedlings, cotyledons, and leaves), *BrFLC2* transcript levels are higher in late flowering pools of DH lines than in pools of early flowering DH lines. *BrFLC2* expression diminished after different durations of seedling vernalization in both early and late DH lines. The reduction of *BrFLC2* expression upon seedling vernalization of both early and late flowering DH lines was strongest at the seedling stage and diminished in subsequent growth stages, which suggests that the commitment to flowering is already set at very early developmental stages. Taken together, these data support the hypothesis that *BrFLC2* is a candidate gene for the flowering time and vernalization response QTL in *B. rapa*.

Key words: *Brassica rapa*, *FLOWERING LOCUS C*, flowering time, quantitative trait loci, vernalization.

Introduction

In flowering plants, the change from vegetative to reproductive development is a major transition that is sensitive to various seasonal climatic signals (Koornneef *et al.*, 2004). Controlling the timing of this transition is especially important in crop plants to ensure high agricultural productivity.

Many genes that control flowering time have been identified by analysing *Arabidopsis* mutants (Boss *et al.*, 2004; Koornneef *et al.*, 2004). These studies have shown that in *Arabidopsis* multiple pathways are involved in controlling flowering time, including the vernalization, photoperiod, autonomous, and gibberellin pathways (Mouradov *et al.*, 2002; Jack, 2004; Schmitz and Amasino, 2007; Alexandre and Hennig, 2008; Seo *et al.*, 2009). The largest difference in flowering time among *Arabidopsis* ecotypes appears to be due to allelic variation at the *FLC* (*FLOWERING LOCUS C*) and *FRI* (*FRIGIDA*) loci (Koornneef *et al.*, 2004; Engelmann and Purugganan, 2006). *FRI* acts upstream of *FLC* to regulate *FLC* expression positively (Michaels and Amasino, 1999). *FLC* encodes a MADS-box transcription factor that functions as a repressor of flowering time in the vernalization pathway (Sheldon *et al.*, 1999, 2000, 2008; Schmitz and Amasino, 2007). Genetic analyses have revealed that the difference in flowering time between early and late flowering ecotypes is largely dependent on allelic variation in *FLC* (Michaels and Amasino, 1999; De Lucia *et al.*, 2008).

Vernalization represses the expression of *FLC* and promotes flowering in vernalization-responsive late flowering

Arabidopsis, and this repressed state is stable during plant development, while the gene is reset to an active transcriptional state in the next sexual generation (Michaels and Amasino, 1999; Sheldon *et al.*, 2000; Schmitz and Amasino, 2007). The extent of promotion of flowering by cold is proportional to the duration of cold exposure; shorter periods of cold exposure result in a smaller acceleration of flowering time and only partial repression of *FLC* expression (Sheldon *et al.*, 2000).

The *Brassica* genus, comprising a large and diverse group of important oil, vegetable, fodder, and condiment crops, is closely related to *Arabidopsis thaliana*, which is a member of the Brassicaceae family. Cultivated varieties of the diploid species *Brassica rapa* can differ based on morphological appearance and consumed organs, which include leafy types, turnip types, and oil types, and exhibit a diverse range of developmental and morphological traits (Zhao *et al.*, 2005). Flowering time is a very important developmental trait, and wide variation exits among *B. rapa* accessions, and their flowering habits are generally controlled by temperature and/or day length. Vernalization is the acquisition of the competence to flower by exposure to prolonged periods of cold or low temperatures. *Brassica rapa*, in contrast to *B. oleracea* (Lin *et al.*, 2005), can be vernalized at the germinated seed stage, which can shorten the generation time and thus speed up genetic research. However, the vernalization response is different among the different cultivar groups. In general, oil types and several pakchoi cultivars flower very early even under non-vernalized conditions. The very late flowering types are mainly Chinese cabbages and turnips, which need long-term vernalization to accelerate flowering.

In previous QTL (quantitative trait locus) analyses of flowering time in *Brassica*, evidence has been presented for a role for *FLC* genes as candidates underlying the flowering time QTL in *B. napus*, *B. oleracea*, and *B. rapa* (Teutonico and Osborn, 1995; Osborn *et al.*, 1997; Tagede *et al.*, 2001; Schranz *et al.*, 2002; Lou *et al.*, 2007; Okazaki *et al.*, 2007). A number of *FLC* paralogues in *B. rapa* (*BrFLC1*, *BrFLC2*, *BrFLC3*, and *BrFLC5*) have been cloned, and mapped both using *in situ* hybridization and genetically (Schranz *et al.*, 2002; Kim *et al.*, 2006; Yang *et al.*, 2006). The study of *BrFLC* expression in Chinese cabbage (Kim *et al.*, 2007) and *B. napus* (Tadege *et al.*, 2001) indicates that the *Brassica FLC* genes act similarly to *Arabidopsis FLC*. A major QTL with *BrFLC2* as the candidate gene on A02 was identified in several *B. rapa* populations evaluated in different environments, locations, and seasons (Lou *et al.*, 2007). In another study, the analysis of sequence variation of *BrFLC1* on A10 suggested that a naturally occurring splicing mutation in the *BrFLC1* gene is associated with flowering time variation in *B. rapa* (Yuan *et al.*, 2009).

From the above it is postulated that the different *FLC* paralogues in *B. rapa* play a role in modulating flowering time and the vernalization response. This paper addresses the effect of vernalization on flowering time in a double haploid (DH) population from a cross between an early flowering yellow sarson and a pakchoi accession and on *BrFLC2* expression in selected lines of this DH population. The possible roles of *BrFLC2* in flowering time regulation and vernalization response are discussed.

Materials and methods

Plant materials and growth conditions

DH population 38 was established from a cross between pakchoi PC-175 (cultivar: Nai Bai Cai; accession number: VO2B0226) and yellow sarson YS-143 (accession number: FIL500) (Lou *et al.*, 2008). A total of 71 lines from population DH38 were evaluated for flowering time under vernalized conditions. Seedlings were germinated on wet filter paper at 25 °C, thereafter vernalized in the dark at 5 °C for 18 d or 31 d, and all transplanted to soil (pots of Ø 17 cm) at the same time. Plants were grown at 24/18 °C (day/night) with a 16 h photoperiod in a greenhouse during spring (February to May) of 2007 in Wageningen University. The two sets of 71 DH lines (vernalized for 18 d and 31 d) were grown in a randomized complete design across three blocks.

The same set of DH lines was also evaluated for flowering time under non-vernalized conditions during winter 2004 (FL04wi), autumn 2005 (FL05au), and spring 2005 (FL05sp). Wide variation existed for flowering time within population DH38 and between the different seasons (Lou *et al.*, 2007). Based on these flowering time data and QTL analyses together with the graphical genotype data of DH lines of population DH38, five early and five late DH lines with and without the A02 QTL, respectively, and the two parental lines (PC-175 and YS-143) were selected for further analysis (Table 1). The expression of *BrFLC2* mRNA was studied in these genotypes after six different vernalization treatments. The germinated seeds of selected DH lines and the parents of the population were vernalized at 5 °C in the dark for 31, 21, 11, 5, 2 and 0 d. Then six seeds per genotype were simultaneously transferred to soil (pots of Ø 17 cm), placed in a climate room, and grown under a 12 h photoperiod at 20/18 °C (light/dark).

Flowering time evaluation

In the experiment of flowering time QTL analysis, the number of days to flowering (FL) of the DH38 plants after vernalization for 18 d (FL07sp-v18) or 31 d (FL07sp-v31) was measured from transplantation into the greenhouse until the first flower opened. The vernalization response (VR) was calculated based on the difference in days to flowering between this experiment (FL07sp-v) and the experiment of spring 2005 (FL05sp) under non-vernalized conditions (Lou *et al.*, 2007), VR-v31=FL05sp–FL07sp-v31, VR-v18=FL05sp–FL07sp-v18.

After collecting samples for RNA isolation from plants of six vernalization treatments (from germinated seeds until 7 d after the seventh leaf stage), 2–4 plants per DH line and parental line were transferred from the climate room to a greenhouse and grown under a 16 h photoperiod at 24/18 °C (day/night) during autumn 2007 (September–October). The flowering time was evaluated for each plant from transplantation into soil until the first flower opened.

QTL analysis

The linkage maps of DH38 have been constructed by the JoinMap program 4.0 based on amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers (Lou *et al.*, 2008). *BrFLC2* was mapped on A02 using SSR markers, and co-localized with the major flowering time QTL. Candidate QTLs that affect traits of interest were identified by interval mapping (IM) and verified by multiple-QTL model mapping (MQM) methods using the software MAPQTL 5.0 (Van Ooijen, 2004). The analysis started with the IM test to find putative QTLs. MQM analysis was then performed to locate QTLs precisely after the

Table 1. The flowering time and genotypic data in the region of the flowering time QTL on chromosome A02 for the 10 selected DH lines

Above the line the flowering times (days) observed in previous experiments (FL04wi, FL05sp, FL05au) and in this study (FL07sp-v31, FL07sp-v18) are listed; below the line the marker genotypes of a 40 cM region around marker BrFLC2 underlying the flowering time QTL on chromosome A02 are given with map positions. x, yellow sarson YS-143 allele; y, pakchoi PC-175 allele.

Trait/marker	Experiment/position (cM)[a]	Early lines					Late lines				
		44	23	57	97	151	31	36	90	134	127
Flowering time	FL07sp-v31	30.3	30.7	30.3	33.3	30.0	40.7	37.7	45.7	41.3	44.0
Flowering time	FL07sp-v18	30.0	30.3	29.0	32.3	27.0	39.0	31.0	40.7	31.7	44.3
Flowering time	FL04wi[b]	48.0	48.0	48.0	39.0	46.0	124.0	118.0	114.0	124.0	108.0
Flowering time	FL05sp[b]	42.0	42.0	35.0	45.0	39.0	67.0	67.0	74.0	80.0	–
Flowering time	FL05au[b]	43.0	39.0	40.0	41.0	42.0	59.0	57.0	58.0	–	63.0
Mya2MSE1M321	13.41	x	x	x	x	x	y	–	y	x	x
BrFLC2	34.84	x	x	x	x	x	y	y	y	y	y
P23M47M115.6	43.45	x	–	x	x	x	y	y	y	y	y
E39M20M368.3	51.01	x	–	x	x	x	y	y	y	y	y
E39M20M360.0	51.45	x	–	x	x	x	y	y	y	y	y
E34M15M142.8	54.01	x	y	x	x	x	y	y	y	y	y
E34M16M122.2	55.79	x	x	x	x	x	y	y	y	y	y

[a] FL07sp-v31, flowering time after 31 d vernalization in spring 2007; FL07sp-v18, flowering time after18 d vernalization in spring 2007.
[b] Data from Lou *et al.* (2007). FL04wi, flowering time in winter 2004; FL05sp, flowering time in spring 2005; FL05au, flowering time in autumn 2005.

automatic selection of cofactors in the vicinity of the QTL. Only significant markers at P <0.02 were used as cofactors in the multiple QTL detection. A map interval of 5 cM was used for both IM and MQM analyses. A permutation test was applied to each data set (1000 repetitions) to decide the LOD (logarithm of odds) thresholds (P=0.05). In this study, a LOD value of 3.00 was used as a significant threshold. QTLs were graphically displayed using Map chart 2.2 (Voorrips, 2002).

RNA isolation

Material for RNA isolation was harvested from several tissues at several developmental stages (12 h photoperiod at 20/18 °C). Supplementary Table S1 available at *JXB* online lists the different tissue samples and collection times/plant developmental stages that were harvested for RNA extraction. Plant samples were collected at different developmental stages: seedling stage (germinated seeds with sprout before transplanting, after vernalization treatment); third leaf stage (cotyledons and first and second leaf), fifth leaf stage (first and second leaf and third and fourth leaf), seventh leaf stage (first and second leaf and third and fourth leaf), and the latest stage, which is 7 d after the seventh leaf stage (first and second leaf and third and fourth leaf). For the seedling stage, three germinated seeds with sprout were collected for each genotype after vernalization. For other developmental stages, the samples were collected from one different individual plant per DH line or parental genotype. At the latest collection stage 7 d after the seventh leaf stage, plants of early DH lines and YS-143 already had flower buds.

Total RNA was extracted using TRIZOL reagent (Invitrogen) starting with ~300 mg of leaf powder. RNA concentration and purity were determined with Nanodrop, and quality was checked by agarose gel electrophoresis. Total RNA (5 μg) of all samples was treated with DNase I (Invitrogen) according to the manufacturer's instructions. DNA-free total RNAs of the early and late DH lines were pooled in equal concentrations and thereafter both parental and pool RNA were converted into cDNA using oligo(dT) primers, dithiothreitol (DTT; 0.1 M), RNaseOUT, and Superscript II (Invitrogen). cDNA was quantified and used for semi-quantitative reverse transcription-PCR (semi-quantitative RT-PCR) and for real-time RT-PCR.

Semi-quantitative RT-PCR and quantitative real-time RT-PCR

For semi-quantitative RT-PCR, two pairs of *BrFLC2*-specific primers (FLC2$^{e4\text{-}7}$, forward 5′-AGTAAGCTTGTGGAATCAAATTCTG-3′, reverse 5′-TAATTAAGYAGYGGGAGAGTYAC-3′; FLC2$^{e1\text{-}4}$, forward 5′ CAAGCGAATTGAGAACAAAA-3′, reverse 5′-GAGTCGACGCTTACATCAGA-3′) were selected based on previous reports by Schranz *et al.* (2002) and Kim *et al.* (2007), respectively (Fig. 1). The PCR program used was: 94 °C for 3 min, 30 cycles of 94 °C for 1 min, annealing at a temperature of 55 °C for 1 min, 72 °C for 1 min 30 s, and a final extension at 72 °C for 7 min.

BrFLC2 gene expression was quantified in the early and late pools of DH lines and the parents of population DH38 using quantitative real-time PCR (qRT-PCR). *FLC* coding sequences from *B. rapa* (AY115675, AY115677, AY115678, AY205317, and AY205318) were joined and aligned with the *A. thaliana* mRNA sequence (AF116527). The presence of different homologues of *FLC* (*BrFLC1*, *BrFLC2*, *BrFLC3*, and *BrFLC5*) in *B. rapa* limited the choice for *BrFLC2*-specific primer selection to exon 4, which is variable (Schranz *et al.*, 2002) between different *FLC* paralogues. In this study, *BrFLC2* gene-specific primers (FLC2$^{e3/4\text{–}5/6}$: forward 5′-CTTGTCGAAAGTAAGCTTGTGGAAT-3′; reverse 5′-GCATTTTCTCCTTTTCTTTGAGG-3′) were designed in the junction of exon 3–4 and exon 5–6 with an amplicon size of 150 bp (Fig. 1). The efficiency of cDNA synthesis was assessed by using the constitutively expressed gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (forward 5′-AGAGCCGCTTCCTTCAACATCATT-3′, reverse 5′-TGGGCACACGGAAGGACATACC-3′) which is frequently used as the reference gene in expression studies (Broekgaarden *et al.*, 2007).

QRT-PCR analysis was done with the MyiQ single-colour real-time PCR detection system (Bio-RAD, Veenendaal, The Netherlands) using SYBR green to monitor double-stranded DNA synthesis. Each reaction contained 10 μl of 2× IQ SYBR Green super mix reagent (Bio-RAD), 10 ng of cDNA, and 300 nM of gene-pecific primer in a final volume of 20 μl. The thermal cycling consisted of 95 °C for 30 s, 95 °C for 3 min, and 40 cycles of 95 °C for 10 s and 52 °C for 45 s. After the PCR a melting curve was generated to check the specificity of the amplified fragment. CT (threshold cycle) values were calculated using optical system software, version 2.0 for MYIQ (Bio-RAD). Subsequently, CT

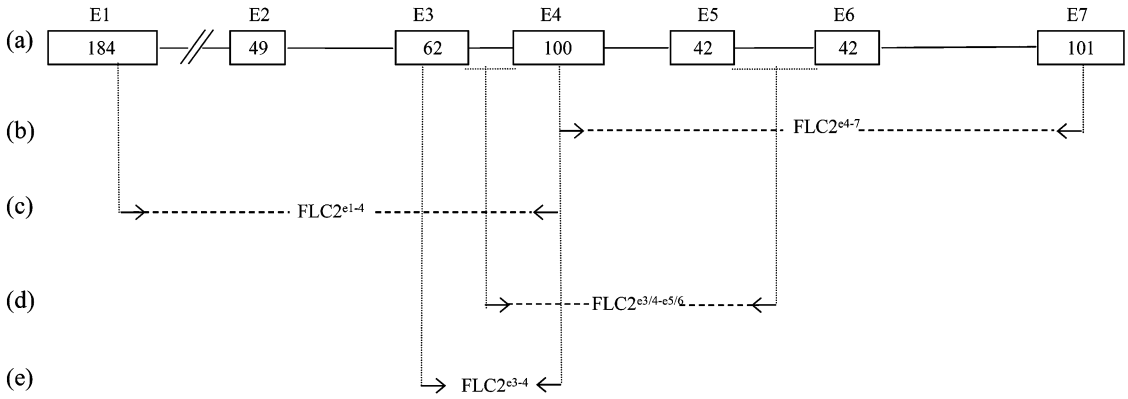

Fig. 1. The *BrFLC2* primers used in this study. (a) Genomic structure of the *A. thaliana FLC* gene copied from Schranz *et al.* (2002). E, exon; the exon size is indicated in the white boxes. (b) FLC2$^{e4\text{-}7}$: *FLC2*-specific primers in exon 4 and exon 7 (Schranz *et al.*, 2002). (c) FLC2$^{e1\text{-}4}$: *FLC2*-specific primers in exon 1 and exon 4 (Kim *et al.*, 2007). (d) FLC2$^{e3/4\text{–}e5/6}$: *FLC2*-specific primers in the junction of exon 3–4 and exon 5–6. (e) FLC2$^{e3\text{-}4}$: *FLC2* intron 3 primers in exon 3 and exon 4.

values were normalized for differences in cDNA synthesis by subtracting the CT value of *GAPDH* from the CT value of *BrFLC2*. Again these values are normalized with a calibrator sample, which was the CT value of *BrFLC2* from the fifth leaf stage (0 day vernalization, late line third and fourth leaf together). Fold change (2^n) in gene expression was calculated by using the $-\Delta\Delta C_T$ method given by Livak and Schmittgen (2001).

Cloning and sequencing of semi-quantitative RT-PCR products

Semi-quantitative RT-PCR with primers FLC2$^{e1\text{-}4}$ resulted in an amplification product of the expected size (~320 bp) and a larger extra band (~400 bp) in pools of early DH lines. Intron 3 of *FLC2* from early and late pools was amplified from genomic DNA by using the primers in exon 3 and exon 4 (FLC2$^{e3\text{-}4}$: forward 5'-AAGTATGGTTCACACCATGAG-3'; reverse 5'-GAGTCGACGCTTACATCAGA-3'). The qRT-PCR product of the unexpected smaller size (79 bp) that was amplified with qRT-PCR primers in samples of pools of early DH lines at the third leaf stage was also sequenced.

The PCR products were excised from agarose gels, purified by QIAquick gel extraction kits (Qiagen), and cloned into PGEM-T easy vector (Promega Corp.). *Escherichia coli* strain DH5α (Invitrogen) was transformed with these constructs and the positive colonies were selected. Plasmid DNA was isolated and sequenced using corresponding primers and aligned in CLASTALV.

Results

QTL mapping

The variation in flowering time of vernalized plants in this experiment (FL07sp-v), of non-vernalized plants in the experiment of spring 2005 (FL05sp), and the variation in the vernalization response for parental lines and corresponding populations of DH38 is shown in Table 2. As can be seen, the mean value of flowering time is enormously reduced in DH38 after vernalization for 18 d and 31 d. After vernalization for 18 d or 31 d, respectively, the variation in days to flowering of DH population was still large (27–70 or 27–61), while the difference between parental lines was only 4 d (32 and 36) or 1 d (29 and 30). Transgression within the population for days to flowering was observed mainly towards late flowering. The vernalization response was also calculated, which had a similar range after 18 d (5–40) compared with after 31 d (5–45).

Table 2. Phenotypic values of flowering time (FL) and vernalization response (VR) of parental lines and DH38

Trait	Parental lines		DH-38 (PC-175×YS-143)	
	YS-143	PC-175	Mean	Range
FL05sp[a]	45.0	54.0	58.2	35.0–87.0
FL07sp-v18	32.0	36.4	37.7	27.0–70.0
FL07sp-v31	28.5	29.9	31.8	27.0–61.0
VR-v18	13.0	17.6	20.2	4.7–39.3
VR-v31	16.5	24.1	20.5	5.3–44.7

[a] Data from Lou *et al.* (2007).

In total, three QTLs for flowering time (FLQTLs) and two QTLs for vernalization response (VRQTLs) were detected in DH38 on two linkage groups (Table 3). One major QTL, FLQTL-1, on the top of A02 for flowering time in non-vernalized experiments (FL05sp) was detected and explained 64% of the variation (Table 3). After 18 d vernalization, FLQTL-2 was detected at the same position on A02, explaining 30.9% of the variation for flowering time. After 31 d of vernalization the FLQTL on A02 disappeared (Fig. 2). One minor flowering time QTL explaining 15% of the variation (FLQTL-3) in the experiment with 18 d vernalization was detected on A08; this QTL was not found in non-vernalization experiments. Two QTLs for the 18 d and 31 d vernalization response (VRQTL-1 and VRQTL-2) were detected on the top of A02, which explain 72% and 77% of the variation, respectively, and appear to be the main loci responsible for the vernalization response in this population.

Flowering time response after different vernalization periods

For further analysis of the effect of vernalization on flowering time, five early and five late DH lines of population

Table 3. Results of QTL analysis of flowering time and vernalization response in DH38 population

QTL	Trait	Linkage group	Position	LOD	%EXP
FLQTL-1	FL05sp[a]	A02	34.8	11.6	63.8
FLQTL-2	FL07sp-v18	A02	34.8	5.6	30.9
FLQTL-3	FL07sp-v18	A08	60.1	3.1	15
VRQTL-1	VR-v18	A02	34.8	10.8	71.9
VRQTL-2	VR-v31	A02	34.8	13.2	77.1

[a] Data from Lou *et al.* (2007).

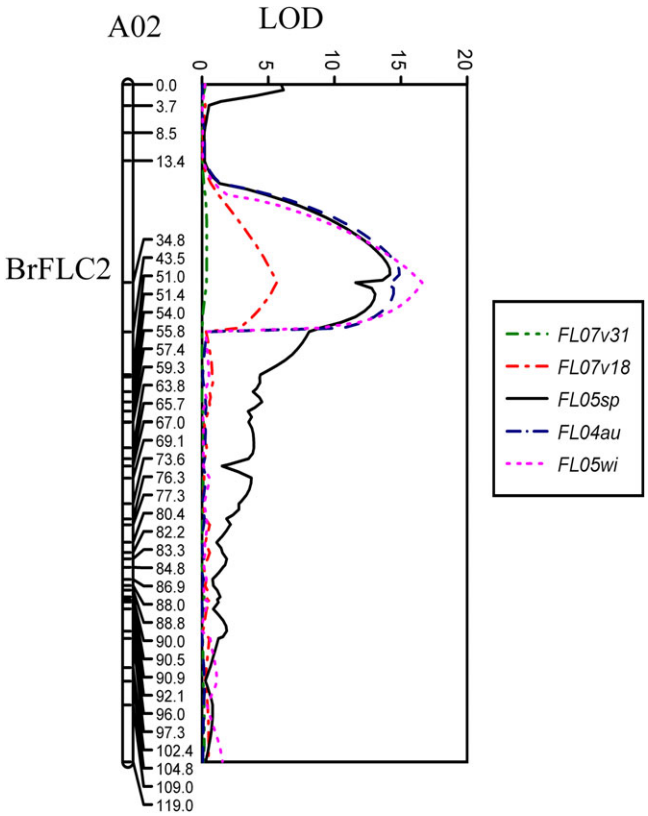

Fig. 2. The LOD profiling of the flowering time QTL on A02 in population DH38 under different experimental conditions.

DH38 were selected (Table 1); early lines had yellow sarson alleles at the flowering time QTL region on A02, while late lines had pakchoi alleles. Without vernalization the early lines flowered after an average of 58 d, while late lines flowered after 98 d (Fig. 3). Upon vernalization for 31 d, early lines flowered after 32 d and late lines after 56 d, which is a decrease of ~30 d. The yellow sarson YS-143 parent and early pools showed a similar reduction in flowering time, whereas flowering time in late lines is transgressive over the pakchoi PC-175 parent, with a similar reduction upon vernalization. The biggest decrease in flowering time occurred between 5 d and 11 d of vernalization in late DH lines and the PC-175 parent.

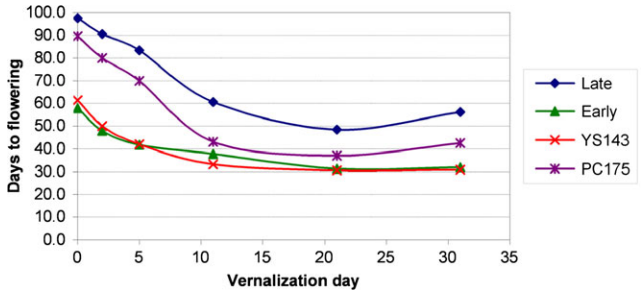

Fig. 3. Effect of vernalization (0, 2, 5, 11, 21, and 31 d) on flowering time in pools of selected DH38 lines (five lines in the early pool, five lines in the late pool) and parents yellow sarson (YS-143) and pakchoi (PC-175). Flowering time is expressed as 'days to flowering' from the transplanting date to flower opening. The average flowering times of five DH lines with three plants per pool are presented and the average flowering times of three parental plants are given.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was conducted with *BrFLC2*-specific primers ($FLC2^{e4\text{-}7}$ and $FLC2^{e1\text{-}4}$) in pools of early and late DH lines and parental lines to quantify *BrFLC2* expression in different developmental stages and tissues (seedlings, cotyledons, or leaves) after different vernalization periods. As seen in Fig. 4, *BrFLC2* transcript was more abundant in late pools compared with early pools in all stages/tissues. Reduction in flowering time after vernalization also correlated with the decrease in *BrFLC2* transcript as indicated by semi-quantitative RT-PCR (Fig. 4A).

In RT-PCR products using primers $FLC2^{e1\text{-}4}$ of first and second leaves harvested from fifth leaf stage plants, an extra larger band (400 bp) appeared in addition to the expected band of 320 bp in the early pools (Fig. 4B). This 400 bp fragment was cloned and sequenced and the sequences were aligned to known *FLC* sequences. This showed that intron 3 of 86 bp was retained, as a result of alternative splicing with intron retention (Supplementary Fig. S1 at *JXB* online). Alternative splicing was clear in early lines in all developmental stages, whereas in the seedling stage alternative splicing was also observed in late pools and in the pakchoi parent (PC-175) (Supplementary Fig. S2 at *JXB* online). Intron 3 was amplified from both early and late pools using primers $FLC2^{3\text{-}4}$ and cloned. Sequence analysis of intron 3 showed that the sequences were identical in both late and early pools (Supplementary Fig. S3 at *JXB* online).

Quantification of BrFLC2 gene expression by qRT-PCR

Regulation of *BrFLC2* gene expression and flowering time by vernalization was further studied by qRT-PCR. To avoid amplification and quantification of several alternatively spliced transcripts, specific primers were designed over exon–exon junctions ($FLC2^{e3/4\text{–}5/6}$: forward, 11 bp in exon 3 and 14 bp in exon 4; reverse, 14 bp in exon 5 and 9 bp in exon 6) to amplify only *BrFLC2* mRNA with properly spliced intron 3. The expected semi-quantitative

RT-PCR product size and sequence data indicated that FLC2$^{e3/4–5/6}$ primers are indeed *FLC2* specific.

Quantitative *BrFLC2* expression in different tissues and from different developmental stages after different vernalization treatments of parents and pools of late and early DH lines is shown in Fig. 5 and Supplementary Fig. S4 at *JXB* online. The differences in *BrFLC2* expression between vernalized and non-vernalized plants are the largest in the seedling stage and slowly diminish. In the late pools of DH lines, the level of *BrFLC2* expression decreases with vernalization time, and this effect is indeed the clearest in the seedling stage, with a 32- (2^5) fold decrease in vernalized seedlings for 31 d compared with non-vernalized seedlings. *BrFLC2* expression is correlated with vernalization duration. During plant development, the difference in *BrFLC2* levels in similar tissues exposed to different durations of vernalization decreases. In leaves of plants in the third leaf stage, the difference in *BrFLC2* expression between non-vernalized plants and plants vernalized for 31 d, is >16- (2^3) fold and at later stages these differences diminish to only 2-fold. Figure 5 also shows that during plant development from seedling to seventh leaf stage *BrFLC2* levels increase 16- (2^4) fold in plants vernalized for 31 d, while for plants vernalized for 0–5 d there is almost no increased expression.

In the pools of early lines the same trends can be detected. In the early DH pools the level of *BrFLC2* expression also decreases with vernalization time and this effect is again clearest in the seedling stage. *BrFLC2* expression in seedlings vernalized for 31 d is 64 (2^6) times lower than that in non-vernalized seedlings. Also here *BrFLC2* expression is correlated with vernalization duration, even though the seedlings of the 21 d vernalization treatment have a rather high *BrFLC2* expression. In the early lines the difference in *BrFLC2* levels in similar tissues exposed to different periods of vernalization again decrease during plant development. In leaves of plants in the third leaf stage, the difference in *BrFLC2* expression between non-vernalized plants and plants vernalized for 31 d is decreased to 32- (2^4) fold but at later stages the decrease in expression still varies between 4- and 16-fold, which is more than in the late pools of DH lines. Again during plant development from seedling to seventh leaf stage *BrFLC2* levels increase 8- (2^3) fold for plants vernalized for 31 d, while in non-vernalized plants there is no increased expression during plant development. In Supplementary Fig. S4 at *JXB* online, the RNA levels of parents YS-143 and PC-175 are also presented; the results are very similar to those of the pools of DH lines.

There were some unexpected expression levels: for example unexpected high values in the third leaf stage of early pools (cotyledons, 11 d vernalization; first and second leaves, 5 d vernalization) and unexpected low values in the

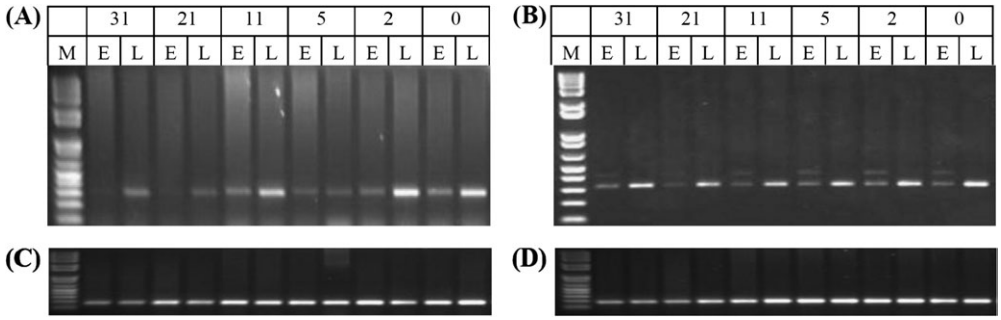

Fig. 4. Semi-quantitative RT-PCR using *BrFLC2*-specific primers (FLC2^{e4-7} and FLC2^{e1-4}) in pooled samples (fifth leaf stage; first and second leaves) of early and late pools of DH lines vernalized for 0, 2, 5, 11, 21, and 31 d. (A) FLC2^{e4-7}; (B) FLC2^{e1-4}; (C) control gene GAPDH; (D) control gene GAPDH. E, early pool; L, late pool; M, 1 kb ladder.

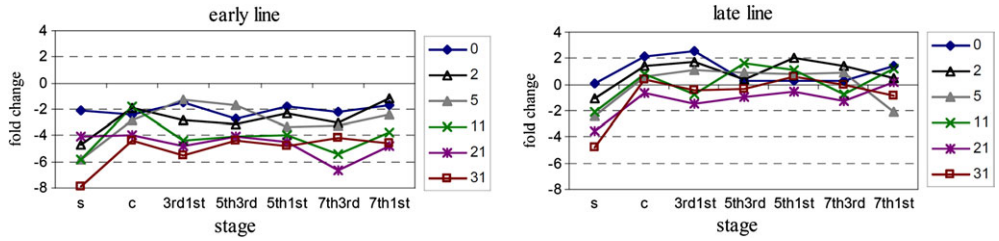

Fig. 5. *BrFLC2* expression (fold change, 2^n) in early and late pools of DH lines during development from seedling to seventh leaf stage plants for the different vernalization treatments (0, 2, 5, 11, 21, and 31 d). Fold change (2^n) was obtained by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) by using a calibrator sample from the fifth leaf stage (third and fourth leaves) of the late pool of DH lines without vernalization. s, seedling stage; c, cotyledons of plants in the third leaf stage; 3rd1st, first and second leaves together of a plant in the third leaf stage; 5th3rd, third and fourth leaves together of a plant in the fifth leaf stage; 5th1st, first and second leaves together of plant in the fifth leaf stage; 7th3rd, third and fourth leaves together of a plant in the seventh leaf stage; 7th1st, first and second leaves together of a plant in the seventh leaf stage.

seventh leaf stage of late pools (first and second leaves, 5 d vernalization).

The melting curve of samples collected from third leaf stage plants of early DH pools was irregular compared with that of other stages and pointed to the existence of yet another type of alternative splicing. The sequencing of this transcript (79 bp) from early pools showed that 59 bp of exon 4 and 22 bp of exon 5 were excised; so yet another alternative splicing type (Supplementary Fig. S5 at *JXB* online).

Discussion

The evaluation of flowering time in different seasons and growing conditions allows evaluation of the expression of QTLs in different environments. To identify the genetic regulation of variation in flowering time and the effect of vernalization, QTL mapping was performed for flowering time and vernalization response in a DH population. The major flowering time QTL, FLQTL-1, on A02 clearly decreases upon vernalization. It explains 63.8% of phenotypic variation in the experiment of spring 2005 in DH38 compared with 30.9% in spring 2007 after 18 d vernalization (FLQTL-2), and was not detected after 31 d vernalization (spring 2007). A flowering time QTL explaining 45% of the variation was also detected at the same position on A02 in a large DH population DH68 (165 individuals) derived from a reciprocal cross between YS-143 and PC-175 (data not shown). Since *FLC* is described to be repressed upon vernalization (Koornneef *et al.*, 2004; Sheldon *et al.*, 2008) and *BrFLC2* co-segregates with the flowering time and vernalization response QTL on the top of A02 in this study, it is suggested that *BrFLC2* is a candidate gene for this QTL in the DH population used in this study. To study the role of *BrFLC2*, pools of late and early flowering DH lines were composed based on flowering time and only PC-175 or YS-143 alleles at the A02 QTL, respectively, while the lines in each pool had either pakchoi PC-175 or yellow sarson YS-143 alleles at the *BrFLC1* locus on A10. To investigate the function of alleles of other flowering time QTLs or alleles of flowering time genes on vernalization, different pools have to be formed selected from larger populations, such as the DH68 population.

Earlier experiments of Sheldon *et al.* (1999, 2000) showed that vernalization promotes flowering by reducing the level of *FLC* transcript and protein. We observed a clear down-regulation of *BrFLC2* by vernalization, which is in agreement with results obtained with *FLC* not only in *A. thaliana*, but also in other Brassicaceae species such as *B. napus*, *B. oleracea*, *B. rapa*, and *Thellungiella halophila* (Tadege *et al.*, 2001; Lin *et al.*, 2005; Fang *et al.*, 2006; Kim *et al.*, 2007) and, more recently, outside this plant family (Reeves *et al.*, 2007). Vernalization reduced the expression of the *BrFLC2* gene by reducing the quantity of transcript, which correlated to a delay of flowering in both the early and late pools. In all developmental stages late pools showed more *BrFLC2* transcript than early pools.

The expression of genes that indicate commitment to flowering is already apparent at a very early stage, with transcription peaks in the seedling stage (Kobayashi *et al.*, 1999). Gendall *et al.* (2001) and Levy *et al.* (2002) described two phases in the repression of *FLC* by vernalization; an initial reduction of *FLC* activity that occurs during the cold exposure, and a subsequent maintenance of the repressed state during growth and development of the plant after the end of the cold exposure. In the present experiment, quantitative reduction of *BrFLC2* expression upon seedling vernalization is strongest in the seedling stage and diminishes in subsequent growth stages, which indicates that in *B. rapa* the decision for flower initiation is already set in the earliest developmental stages (seedling). Maintenance of the repressed state is not observed in the present experiment, since *BrFLC2* levels already increased significantly in plants of the third leaf stage and the difference in *BrFLC2* level between leaves of vernalized and non-vernalized plants decreased in later stages, especially in the late flowering pools.

The difference in flowering time under non-vernalized conditions between both early and late lines and their parents was greatly reduced after 11 d of vernalization, which was caused by a larger reduction in flowering time in late pools and PC-175. After 31 d of vernalization, in the late pool flowering time was reduced by 42 d while the *BrFLC2* transcript decreased 32-fold, whereas in the early pool flowering time was reduced by 30 d with a 64-fold decrease in *BrFLC2* transcript.

Analysis of *Arabidopsis* expressed sequence tags (ESTs) showed an unusually high fraction of retained introns (>30%) that may play a regulatory role (Ner-Gaon *et al.*, 2004). The distribution of the transcripts with retained introns is skewed towards stress responses, like a cold treatment as used to vernalize seedlings. Macknight *et al.* (2002) showed that the alternative splicing of the *FCA* transcript has functional significance related to its role in the promotion of floral transition. Short transcripts due to alternative splicing of *FLC* were observed in *Arabidopsis* haplogroup *FLCA* and *FLCB* after a vernalization treatment of 15 d, which were not observed at high levels in plants grown at normal temperature for 15 d (Caicedo *et al.*, 2004). The repression of *FLC* by cold treatment is also associated with changes in histone modification (Sung and Amasino, 2004; Finnegan *et al.*, 2005). Previous studies reported regulation of *FLC* by the regions in its first intron (Sheldon *et al.*, 2002; He *et al.*, 2003), polymorphisms in which led to differential expression and differential splicing patterns. In the present experiment vernalization/cold stress resulted in differences in *BrFLC2* expression that may be partly regulated by alternative splicing, which was detected for intron 3 in all stages in early pools and only in the very early stages of late pools. Real-time PCR using a forward primer in exon 4 with a reverse primer over the exon 5–6 junction amplifies both transcripts with a correctly spliced and retained intron 3. In contrast to the real-time PCR results using a forward primer over the exon 3–4 junction, real-time PCR using this exon 4 forward primer detected very little change in *BrFLC2* transcript levels upon vernalization in pools of early lines (data not shown).

In these early lines alternative splicing was obvious, which strongly contributed to the observed decrease in *BrFLC2* transcript with correctly spliced intron 3. In late lines the intron 4 forward primer gave results comparable with those obtained with the primers over the exon 3–4 junction (data not shown), which agrees with the observation that in late lines intron 3 retention hardly occurs. Alternative splicing was not looked at in other parts of the gene not covered by the primers used in this study.

Multiple copies of *B. rapa* genes homologous to flowering time genes of *Arabidopsis* exist, and these multiple functional loci may contribute to the wide variation in flowering time in this species. There are four *FLC* paralogues in *B. rapa*; in this study *BrFLC2* was found as a candidate gene for the large effect flowering time QTL on A02 in population DH38 from a cross of an early oil type and a middle late leafy type. Yuan *et al.* (2009) reported that the *BrFLC1* gene also contributes greatly to flowering time variation in non-vernalized *B. rapa*. In a previous study (Lou *et al.*, 2007), co-localization of the flowering time QTL with other possible flowering-related genes in multiple segregating populations was discussed. The roles of the different *FLC* paralogues and other flowering time-related genes and their interactions in *B. rapa* are presently being investigated further.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. The different samples that were harvested for RNA extraction.

Figure S1. Sequencing of RT-PCR products in early and late pools using the *BrFLC2*-specific primers $FLC2^{e1\text{-}4}$.

Figure S2. Semi-quantitative RT-PCR using *BrFLC2*-specific primers ($FLC2^{e1\text{-}4}$) in seedling of early and late pools of DH lines and parent lines (P1, YS-143; P2, PC-175) vernalized for 0, 2, 5, 11, 21, and 31 d.

Figure S3. Sequencing of intron 3 from early and late pools using specific primers $FLC2^{e3\text{-}4}$.

Figure S4. Fold change (2^n) in *BrFLC2* expression (fold change) after different vernalization treatments (day) for pools of late and early DH lines.

Figure S5. Sequencing of unexpected smaller size (<100 bp) qRT-PCR products in early pools at the third leaf stage using specific primers $FLC2^{e3/4\text{–}e5/6}$.

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