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ORIGINAL PAPER

Mapping and characterization of *FLC* homologs and QTL analysis of flowering time in *Brassica oleracea*

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Abstract The *FLC* gene product is an inhibitor of flowering in *Arabidopsis*. *FLC* homologs in *Brassica* species are thought to control vernalization. We cloned four *FLC* homologs (*BoFLC*s) from *Brassica oleracea*. Three of these, *BoFLC1*, *BoFLC3* and *BoFLC5*, have been previously characterized. The fourth novel sequence displayed 98% sequence homology to the previously identified gene *BoFLC4*, but also showed 91% homology to *BrFLC2* from *Brassica rapa*. Phylogenetic analysis showed that this clone belongs to the FLC2 clade. Therefore, we designated this gene *BoFLC2*. Based on the segregation of RFLP, SRAP, CAPS, SSR and AFLP loci, a detailed linkage map of *B. oleracea* was constructed in the F_2 progeny obtained from a cross of *B. oleracea* cv. Green Comet (broccoli; non-vernalization type) and *B. oleracea* cv. Reiho (cabbage; vernalization type), which covered 540 cM, 9 major linkage groups. Six quantitative trait loci (QTL) controlling flowering time were detected. *BoFLC1*, *BoFLC3* and *BoFLC5* were not linked to the QTLs controlling flowering time. However, the largest QTL effect was located in the region where *BoFLC2* was mapped. Genotyping of F_2 plants at the *BoFLC2* locus showed that most of the early flowering plants were homozygotes of *BoFLC-GC*, whereas most of the late- and non-flowering plants were homozygotes of *BoFLC-Rei*. The *BoFLC2* homologs present in plants of the non-vernalization type were non-functional, due to a frameshift in exon 4. Moreover, duplications and deletions of *BoFLC2* were detected in broccoli and a rapid cycling line, respectively. These results suggest that *BoFLC2* contributes to the control of flowering time in *B. oleracea*.

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Introduction

Brassica oleracea L. is one of the most important crop species and includes many vegetables such as cabbage, broccoli, Brussels sprouts, kohlrabi, cauliflower and kale. These varieties exhibit a diverse range of flowering times, and their flowering habits are generally controlled by temperature and/or day length (Friend 1985). Low temperature is a key to flowering in many varieties of *Brassica* crops, which can be classified into two types: biennials such as cabbage or kohlrabi, in which flowering is promoted by prolonged exposure to low temperature (the vernalization type), and annuals such as broccoli and cauliflower, which do not require low temperature for flowering (the non-vernalization type).

Genetic information controlling flowering time in *Arabidopsis thaliana* can be transferable to *Brassica* crops because of collinearity and congruence in the *A. thaliana* and *Brassica* genomes (Lagercrantz 1998; Li et al. 2003; Lukens et al. 2003; Suwabe et al. 2006). Flowering responses in *A. thaliana* are regulated by the photoperiodic and autonomous signaling pathways (Devlin and Kay 2000; Mouradov et al. 2002). The *CONSTANS* (*CO*) gene, which encodes a GATA-type transcription factor, plays a key role in photoperiodic regulation of flowering. The autonomous signaling pathway and signals from vernalization are converged with *FLOWERING LOCUS C (FLC)*, which encodes a MADS-box transcription factor that acts as a repressor of the floral transition in a dosage-dependent manner (Michaels and Amasino 1999; Sheldon et al. 1999). Vernalization promotes flowering by reducing levels of the FLC protein, which causes activation of FT and SOC1, which in turn activate flower meristem identity genes to transit vegetable meristem to reproductive phase (Sheldon et al. 1999, 2000; Amasino 2004). In the *A. thaliana* genome, most of the flowering-related genes are mapped to single locus, although some of the genes, such as *CO*, *FLC* and *FY*, are also clustered within a small region of the 5th chromosome (TAIR database, http://www.arabidopsis.org/).

Restriction fragment length polymorphism (RFLP) mapping of quantitative trait loci (QTL) shows that *Brassica* genomes include the chromosome segments equivalent to the top of the 5th chromosome in the *Arabidopsis* genome to which the QTL for flowering time has been mapped (Lagercrantz et al. 1996; Bohuon et al. 1998; Axelsson et al. 2001; Schranz et al. 2002). Examining the flowering of a population without low-temperature treatment, Axelsson et al. (2001) reported that the flowering-time QTLs identified in several *Brassica* species correspond to homologs of *CO*, based on confidence intervals for QTL and map positions for *CO* and *FLC*. In contrast, Kole et al. (2001) and Schranz et al. (2002) provided strong evidence that the flowering-time loci in *B. rapa* are allelic to *FLC* homologs. This discrepancy may be due to differences in the parentage of the populations used to construct linkage maps: Axelsson et al. (2001) used double-haploid (DH) lines developed from F_1 plants produced from a cross of non-vernalization types, whereas Kole et al. (2001) and Schranz et al. (2002) used segregating populations derived from a cross of biennial (vernalization) and annual (non-vernalization) types in *B. rapa*.

Comparative mapping shows that a single locus in the *Arabidopsis* genome is represented by three loci in the diploid *Brassica* genomes (Lagercrantz 1998; Osborn et al. 1997). This may be due to whole-genome triplication followed by chromosome rearrangements, including fusions and fissions that led to the formation of *Brassica* ancestors. Although *Brassica* genomes were rearranged during the process of speciation, each unit genome sustains the genomic structure of *Arabidopsis* (Lagercrantz 1998; Lagercrantz and Lydiate 1996). The number of segmental unit genomes originated from an ancestral species varies from a single copy to multiple copies among *Brassica* genomes. Four *Brassica FLC* homologs (*BrFLC1*, *BrFLC2*, *BrFLC3* and *BrFLC5*) have been identified in *B. rapa*, three (*BoFLC1*, *BoFLC3* and *BoFLC5*) in *B. oleracea* (Schranz et al. 2002) and five (*BnFLC1*, *BnFLC2*, *BnFLC3*, *BnFLC4* and *BnFLC5*) in *B. napus* (Tadege et al. 2001). More recently, a new *FLC* homolog (*BoFLC4*) was isolated from *B. oleracea* (Lin et al. 2005). Redundancy in flowering-related genes like *CO* and *FLC* causes broad variation in flowering period in *Brassica* species (Schranz et al. 2002). In a study of genetic mapping and QTL analysis of *B. rapa*, Schranz et al. (2002) demonstrated that *BrFLC1*, *BrFLC2* and *BrFLC5* play an important role in vernalization and suggested that these *FLCs* act in a dosage-dependent manner to control the flowering response. It is not known which of the four *BoFLCs* identified in *B. oleracea* are important for flowering. Other QTLs that do not correspond to the loci of *CO* and *FLC* also have large effects on flowering time in *B. oleracea* (Camargo and Osborn 1996; Bohuon et al. 1998; Axelsson et al. 2001; Sebastian et al. 2002).

To better understand the genetic control of flowering time in *B. oleracea*, we isolated genomic *BoFLC* genes and also identified QTL controlling flowering time using a linkage map and flowering data from an F_2 population derived from a broccoli DH line by cabbage DH line. The possible role of *BoFLC2* in flowering via vernalization is discussed.

Materials and methods

Plant materials

To analyze flowering time QTL, a DH line (P09) of *B. oleracea* var. *italica* cv. Green Comet (GC; annual cultivar, Takii Seed Co. Ltd., Japan) was crossed with a DH line (P01) of *B. oleracea* var. *capitata* cv. Reiho (Rei; biennial cultivar, Ishi seed company, Japan). F_1 plants were self-pollinated to produce F_2 seeds. The six parental, 5 F_1 and 134 F_2 plants were sown in a greenhouse on 23rd February 2001 and transplanted to the field at the Faculty of Agriculture of Niigata University on 13th April. Monthly mean temperatures during the

growing season were April, 12°C; May, 18°C; June, 21°C and July, 27°C.

To detect a frameshift mutation in exon 4 of *BoFLC2*, cv. Shaster, Green Hat, Height (Takii Seed Co. Ltd.) and Ryokurei (Tohoku Seed Co. Ltd., Japan) of broccoli and cv. Snow Crown, Hakusui (Tohoku Seed Co. Ltd.) and two strains of cauliflower (Kaneko Seed Co. Ltd., Japan) and cv. Neo Ruby of cabbage, and cv. Aojirukale of Kale and cv. Wasekomochi of Brussels sprout (Takii Seed Co. Ltd.) were used.

Cultivar Neo Ruby of cabbage and cv. Shaster of broccoli (Takii Seed Co. Ltd.) and cv. Snow Crown (Tohoku Seed Co. Ltd.) of cauliflower were used to analyze the expression of *FLC* mRNA. The plants were grown in the chamber maintained at 23°C (12 h daylength). Cotyledons and true leaves were collected from 1.5- to 12-week-old-plants.

Measurement of flowering time

Days to flowering (DTF) were measured from transplantation into the field to the first open flower. DTF were recorded for up to 100 days after transplantation. Plants that showed no flowering at the end of the experiment were assigned a value of 130 DTF.

Detection of DNA polymorphism

Total genomic DNA was extracted from young leaves of parental, F_1 and F_2 plants using the CTAB method (Murray and Thompson 1980). To perform RFLP mapping, 2 μg of genomic DNA was digested with *Eco*RI and then applied to a 0.8% agarose gel. After electrophoresis, gels were blotted onto Hybond N membranes (Amersham Biosciences) and probed with *Brassica* DNA clones, which were kindly provided by Professor T. C. Osborn, University of Wisconsin, Madison, WI, USA. RFLP probes were prepared by PCR with digoxigenin using T3 and T7 primers according to the manufacturer's instructions (Roche). Membranes were hybridized at 42°C in a solution containing 50% (v/v) formamide, 5× SSC, 2% blocking reagent (Roche), 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine, 100 μg/ml denatured sonicated salmon sperm DNA and the labeled probes. Filters were washed twice at room temperature in 2× SSC/0.1% SDS for 5 min and twice in 0.1× SSC/0.1% SDS at 68°C for 15 min and were incubated with alkaline phosphatase-conjugated anti-digoxigenin (Roche). DNA was detected by chemiluminescence using CSPD (Roche) according to the manufacturer's instructions.

Polymorphism detection by the sequence-related amplified polymorphism (SRAP) method was conducted according to the method of Li and Quiros (2001), with minor modifications. For amplification, a standard PCR cocktail with the primer pairs listed in Table 1 was used. The first five cycles of PCR were performed at 94°C for 1 min, 35°C for 1 min and 72°C for 1 min, for denaturing, annealing and extension, respectively. The annealing temperature was then raised to 50°C for another 35 cycles. The samples were loaded onto a native 4% polyacrylamide gel and separated at a constant power of 250 V for 2.5 h. The gel was subsequently stained using Gelstar solution (Takara Biomedicals, Japan).

Primer sequences used in cleaved amplified polymorphic sequence (CAPS) analyses are listed in Table 2. These primer sequences were designed based on the structural gene sequences published in the NCBI and TAIR databases, and in the reports of Kuittinen et al. (2002) and Inoue and Nishio (2004). Annealing temperature and extension time for PCR were set according to primer sequence and gene size. For *BoFLC5*, a partial sequence of intron 1 was amplified by the primer pair BoFLC5-33F (5′-AGTGTGGAAGGGATGTGAAAG-3′) and BoFLC5-33R (5′-TGAGGTTACAGACGTCTAAC-3′). The amplicons were digested with one of five restriction enzymes (*Afa*I, *Msp*I, *Mbo*I, *Xsp*I or *Taq*I) and were separated on a native 4% polyacrylamide gel at a constant power

Table 1 Primer pairs used in SRAP

	Forward (5′-3′)		Reverse (5′-3′)
F1[a]	TGAGTCCAAACCGGATA	R1	GACTGCGTACGAATTAAT
F2	TGAGTCCAAACCGGAGC	R2	GACTGCGTACGAATTTGC
F3	TGAGTCCAAACCGGAAT	R3	GACTGCGTACGAATTGAC
F4	TGAGTCCAAACCGGACC	R4	GACTGCGTACGAATTTGA
F5	TGAGTCCAAACCGGAAG	R5	GACTGCGTACGAATTAAC
F11	TACAACGAGTCCGGATA	R11	GATCGTAGCCAATTAAT
F12	TGGATTGGTCCCGGATC	R12	TCGGTCATAGAATTGCT
F13	GGAACCAATCCCGGATG	R14	TACCTAAGCGAATTCAG
F14	AAACTCCGTCCCGGACT	R18	ACTGAGATCCAATTCCG
F18	CGTAAACTCCCCGGCAA	R19	CCCGTTTTTGAATTCTC
F19	TACTGTTGCCCCGGCAT		

[a] F1–F5 and R1–R5 correspond to the me1–me5 and em1–em5 primer pairs reported by Li and Quiros (2001)

Table 2 Primer sequences of CAPS markers for *B. oleracea*

Name	Accession number	Primer sequences (5′-3′)		PCR	CAPS
		Forward	Reverse		
ACS2	AB086353	AGCTACATGCAACAGCCATG	AGTCGTTGTCTTCTTCCTCG	C	P(*Afa*I)
ACT1[b]	AF044573	TGGTTGGGATGAACCAGAAG	CATCAATTCGATCACTCAGG	C	P(*Alu*I)
ALK	AY044425	TCAACTGCTACGCCCTGATT	ACTCGTTTCCCATCTCCTGA	C	P(*Mbo*I)
APETALA	D21125	AATGGTTTATTCAAGAARGCNCA	CGAACGAGTTTGAAAGTRTTYT	C	P(*Msp*I)
APR1	U43412	CCAAGATGTGGGATGAAACT	ACAAGAGGACGCATTACCAC	C	P(*Taq*I)
ASB1[b]	AF195511	CGCAACCCAAGAATGCAATC	ACCAGAACATTCCATCCACT	C	P(*Mbo*I)
BOB26-1	U92651	CTCAGCCGTTCGATCTTTCT	CCAACGGTCATAAACACATG	C	P(*Msp*I)
BOHM13[b]	Z97060	TATGCACTTCCGGTCAGACC	CAGCTTATCTCTCAACTCTG	C	P(*Mbo*I)
BOHM24[b]	U18995	CTTTACCACTGTTTTGGCCG	CTTTCACGAACACAACCTCG	C	P(*Afa*I)
BORED	X64464	GTGGCCAGGCTATCACNTTYGG	AGTCGCTGTGTAGTTTGCYTGNCC	C	P(*Mbo*I)
BTPT	U13632	AGATCTCCCACGATGCAGAG	AGTAAGTCAGCAAAGAGAAC	C	P(*Msp*I)
CAM1	AJ427337	GTTCAAGGAAGCCTTTAGCC	AGAGATAGCTTAGCCGAAGC	C	P(*Alu*I)
CAM2	AJ427338	TGACCGATGACCAGATCTCA	GTCGCAACCGAATCAAGTTC	C	P(*Mbo*I)
CCOL	U18675	CGGTTGGCTTCATACNGGNGA	TCCTCAATATCTTCCCNGWNGG	C	P(*Afa*I)
CHI[a]	M86358	GTGGAAGGGAAAAACTACGGAGGAG	CCGGTTTCAGGGATACTATCATCTT	C	P(*Alu*I)
CO	AF016010	ATGTTCAAACAAGAGAGTAAC	CTTTATTTTTGGCCATAGAAT	C	P (*Alu*I)
DGAT1	AF164434	GGCGATTTTGGATTCTGGAG	CGGTTCATCAGGTCATGGTA	C	P(*Mbo*I)
FLC1	AY115674	GAGGAATCAAATGTCGATAA	CTAATAAAGCAGTGGGAGAG	C	P(*Mbo*I)
FLC2	AF116527	CCATGAGCTACTAGAACTTG	CTAATAAAGCAGTGGGAGAG	C×2	P(*Alu*I)
FLC3	AY115673	GTGGAATCAAATGTCGGTGG	CTAATAAAGCAGTGGGAGAG	C	P(*Alu*I)
FLC5[c]	–	AGTGTGGAAGGGATGTGAAAG	TGAGGTTACAGACGTCTAAC	C	P(*Xsp*I)
FLC5 extra-band	AY115672	TGGAATCAATTGTCGATGTA	CTAATAAAGCAGTGGGAGAG	P	–
GA1[a]	U11034	CAAGGATACCAAAAGAGATAATGC	CGTTTTCTCCACCATATTGATC	C	P(*Afa*I)
GAPB	M64118	GGCTAGAAGTCGCTGAATTC	TGGTAGAGACATCAGAGCAC	C	P(*Mbo*I)
GDH2	AB066298	CGAGAAGAGTCTCATGATCC	CATTAAGCTTCCCAACCACG	C	P(*Msp*I)
GSA1	U03773	ACCAGCTTCTAACCGATGCT	GTATATCCTCGGGAGTGTGA	C	P(*Alu*I)
GSL	AF399834	TGGCATCGTCACTTCTGACA	CTAATGCTACTCGCGACCAT	C×2	P(*Mbo*I)
GTR	AC002333	GACATCATCCARAARCAYCARAC	CTCTCCTTCCATCACTTCCTTA	C	P(*Mbo*I)
IPI[b]	AF236092	ATGCTGTTCAAAGACGCCTC	TACAGCTTCACCGAGAGTTC	C	P(*Mbo*I)
MSI1	AF016846	GAGATCGAGGAGCGACTAAT	TGTTGTCCTCAGCAACACTG	C	P(*Afa*I)
MYR	Z21978	CATAAAGCTTCTTCATGGAC	TCATGCATCAGCGAGCTTCT	C×2	P(*Alu*I)
NDPK3	AB072239	GGTCTTCTCTCAGTTTCACT	AGTTATCGCCATAGATCCAC	C	P(*Msp*I)
NIT	AF380304	ACATCTCTGGAACGTTGCAT	CCTTGAGTAATGTCCGACC	C×2	P(*Map*I)
PGIC[a]	X69195	TCGAACCCGGGAGAGGTAGACCA	TGCTGTCAGCACTAATCTTGCG	C	P(*Alu*I)
PIP1b2[b]	AF299051	GAGACAACCCATCGGAACAT	TGGACTTGAATGGGATGGCT	C	P(*Msp*I)
PLD2[b]	AF090444	GGAGTATCCAAGACGCTTAC	CAGCAGCAATGTAGAGACAG	C	P(*Mbo*I)
SFR2[b]	X98520	TCGGCTACAGAATCTCTCAC	TCTAGATCAGCAGCTGCTAG	C	P(*Alu*I)
SLG	X55275	ATGAAAGGCG TAAGAAAAAC CTA	CCGTGTTTTATTTTAAGAGAAAGAGCT	P	–
TFL1[a]	D87519	GGTTTCACGAGTGGCTTATTCC	CCGTCGTCATCCTCACCTTC	C	P(*Afa*I)
TMT1[b]	AF387791	TCCTGCCTGAAACTGTTGAG	ACCTCCCAAGCTTCTCTTTG	C	P(*Alu*I)

C common bands, ×*2* multiple bands, *P* polymorphic bands

[a] Primer sequences obtained from Kuittinen et al. (2002)

[b] Primer sequences obtained from Inoume and Nishio (2004)

[c] The *FLC5*-specific primer pair was designed based on the first intron of *FLC5*

of 250 V for 2.5 h. The gel was subsequently stained using Gelstar solution (Takara Biomedicals).

Amplified fragment length polymorphism (AFLP) analysis was conducted using the AFLP Analysis System I kit (Life Technologies). Amplification of the microsatellites was performed as reported by Suwaba et al. (2002, 2004).

To detect a frameshift mutation in exon 4 of *BoFLC2*, the DNA fragments were amplified using *BoFLC2*-F3 designed from the sequence of exon 4(5′-GTAAGCTTGTGGAATCAAATTCT-3′) and *BoFLC2*-ex6R designed from the sequence of exon 6 (5′-TGGCTAGCCAAACCCTGGTT-3′) from broccoli and cauliflower. The amplicons were digested with *Alu*I and polymorphism was detected by 4% acrylamide gel electrophoresis.

Cloning of genomic *BoFLC* genes

BoFLC1, *BoFLC3* and *BoFLC5* homologs were amplified from genomic DNA isolated from the GC and the Rei DH line of *B. oleracea* using the gene-specific primers reported in Schranz et al. (2002). To isolate additional genomic *Brassica FLC* genes, the forward primer FLC2-F (5′-CCATGGCTACTAGAACTTG-3′) and the reverse primer FLC-R (5′-CTAATAAAG CAGTGGGAGAG-3′) were designed based on exons 3 and 7, respectively, of *FLC* from *A. thaliana* (AF116527). PCR products were cloned into pBluescript II SK+ (Stratagene) and sequenced using the CEQ8000 Genetic Analysis System (Beckman Coulter, Inc., USA). At least two independent clones from separate PCR reactions were sequenced for *BoFLC2*. The coding sequences for *FLC* from *A. thaliana* (AF116527), *B. napus* (Tadege et al. 2001), *B. oleracea* and *B. rapa* (Schranz et al. 2002; Lin et al. 2005) were aligned and were phylogenetically analyzed by the NJ method using GENETYX software (GENETYX, Japan). The nucleotide sequence data reported in this article have been deposited in GenBank at NCBI with the accession numbers DQ222849 (*BoFLC2*-Reihou) and DQ222850 (*BoFLC2*-GC).

Map construction and QTL analysis

To generate the map, 38 RFLP, 43 CAPS, 56 SRAP, 14 SSR and 36AFLP markers were used. Linkage analyses were performed using the JoinMap program, version 3.0 (Van Ooijen and Voorrips 2001). The segregation of 187 markers was studied, and LOD scores of 4.0 were used in the analysis of linkage groups. The Kosambi mapping function was used to convert recombination frequencies into map distances. The RFLP probes and the SSR markers included in this study had been used in previous mapping studies (Ferreira et al. 1994; Parkin et al. 1995; Sharpe et al. 1995; Bohuon et al. 1998; Sebastian et al. 2000; Udall et al. 2005; Parkin et al. 2006; Suwabe et al. 2006; Kim et al. 2006) and the internationally agreed chromosome nomenclature is available at http://www.brassica.info/information/lg_assigments.htm. According to that information, tentative assignments of linkage groups, O1–O9, were made in the present study.

QTL analysis was performed using a composite interval-mapping analysis (Zeng 1994) with MapQTL version 2.0 (Van Ooijen et al. 2000) and QTL Cartographer version 1.16 (Basten et al. 2002). A forward–backward stepwise regression was performed to choose co-factors before performing QTL detection. A 1,000-permutation test was performed with QTL Cartographer to estimate the appropriate significance threshold for analysis. A LOD threshold of 2.4, corresponding to a genome-wise significance level of 0.10, was chosen.

Southern blot analysis using a *FLC* gene probe

Southern blot analysis was conducted as described above. Aliquots of genomic DNA (2 μg) were digested to completion with *Eco*RI and detected using the *BoFLC2*-specific probe, which contains partial sequences from exons 3 to 7, was labeled with digoxigenin. GC broccoli, Rei cabbage and Choten (Sakata Seed Co. Ltd., Japan) of F_1 cabbage cultivar and rapid-cycling line (CRGC3-1) of *B. oleracea* were used.

RNA extraction and detection of mRNA by RT-PCR

Total RNA was isolated from the cotyledon, the apex, 2nd leaves and 6th leaves from 1.5- to 12-week-old plants using the ISOGEN reagent (Nippongene, Japan). For first-strand cDNA synthesis, 0.5 μg of total RNA was reverse transcribed with a reaction mixture containing 10 U/μl SUPERSCRIPT II reverse transcriptase (Invitrogen, USA), 0.5 mM dNTP mix, 10 mM DTT, 1 U/μl RNase inhibitor (Toyobo, Japan), 10 μM dT17 adapter primer and 1× First-Strand Buffer (Invitrogen) at 42°C for 50 min. PCR was performed as described above for genomic PCR using gene-specific primers. Amplification of actin was used as a control to ensure that equal amounts of cDNA were added to each PCR. The primers used to amplify actin cDNA were 5′-TGGTTGGGATGAACCAGAAG-3′ and 5′-CATCAATTCGATCACTCAGG-3′. The RT-PCR products were separated on a 1.2% agarose gel and stained by ethidium bromide.

Results

Cloning of genomic *BoFLC* genes

To isolate *BoFLC* genes, PCR was conducted using gene-specific primers with genomic DNA from the DH lines of Rei and GC of *B. oleracea* (Fig. 1). PCR products of 1.1 and 1.0 kb were amplified from both GC and Rei DNA using *BoFLC1*- and *BoFLC3*-specific primers, respectively (Fig. 1). These amplified products had high sequence identity to the *BoFLC1* and *BoFLC3* genes reported by Schranz et al. (2002) (data not shown). A 1.4-kb common band was amplified from Rei and GC DNA when the *BoFLC5*-specific primers were used. An additional 1.6-kb band was detected only in GC DNA. The 1.4-kb band was cloned and was identified as *BoFLC5*, based on sequence homology.

Using FLC2-F and FLC-R primer pairs, designed based on sequences from exons 3 and 7 of *FLC*, two bands (1.5 and 1.8 kb) were amplified from both Rei and GC genomic DNA (Fig. 1). The 1.5-kb sequence was identical to that of *BoFLC5*. The clones derived from the 1.8-kb amplicons from Rei and GC had 99% nucleotide sequence identity. A comparison of these cloned sequences to the nucleotide sequences of the *BoFLC* genes reported by Schranz et al. (2002) showed that they contain exons 3–7 with the interspaced four introns (Fig. 2). The coding region derived from the 1.8-kb sequence amplified from Rei DNA contained a single open reading frame. The 1.8-kb sequence derived from GC DNA, however, contained a frameshift that was due to a 1-base deletion in exon 4. Therefore, it is unlikely that this *BoFLC* gene is functional in the GC DH line. Comparison with other *BoFLC* genes revealed that the coding sequences of the 1.8-kb bands had 100% sequence identity with *BoFLC4* that was isolated by Lin et al. (2005), except for the 1-base deletion of GC sequence, although the intron sequences were different from those of *BoFLC4*. The 1.8-kb sequences had 91% sequence identity with *BrFLC2* and 83–87% sequence identity with the remaining *BoFLCs* (*BoFLC1*, *BoFLC3*, *BoFLC5*).

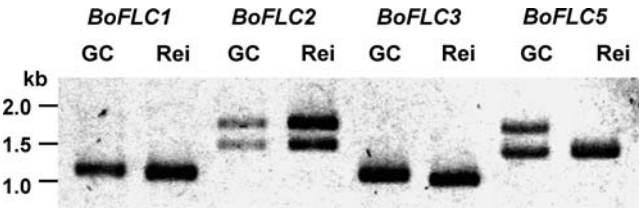

Fig. 1 PCR amplification of *FLC* sequences from genomic DNA isolated from Green Comet (*GC*) and Reiho (*Rei*) DH lines of *B. oleracea*. *BoFLC1*, *BoFLC3* and *BoFLC5* were amplified using gene-specific forward primers designed to a highly variable region of exon 4 (Schranz et al. 2002) and a conserved exon 7 reverse primer (FLC-R). Genomic *BoFLC2* was amplified by using the sequence located in exon 3 (FLC2-F) with FLC-R

The genomic sequence of exon 2 of the 1.8-kb fragment was subsequently obtained by PCR using a primer based on the highly conserved exon 2 with a specific reverse primer designed in exon 7. Phylogenic analysis was conducted using the coding sequence from exons 2–7 (Fig. 3). *FLC* sequences fall into four clades, and *FLC* sequences from the three *Brassica* species are distributed in each clade. The 1.8-kb sequence obtained from Rei was found to be part of a clade including *BrFLC2*. The results indicate that an ancestral *FLC* gene multiplied into four genes, *FLC1*, *FLC2*, *FLC3* and *FLC5*, before the evolution of the three *Brassica* species. Based on this phylogeny, the 1.8-kb sequence obtained from Rei was designated as *BoFLC2- Rei* so that the nomenclature of *BoFLC* reflects *FLC* phylogeny in that each *Brassica FLC* homolog diverged from each gene of the common ancestor.

The polymorphic change on the *Alu*I recognition site in exon 4 of *BoFLC2-Rei* (functional) and *BoFLC2-GC* (non-functional), which was produced by the mutational change of a 1-base deletion in exon 4 of *BoFLC2-GC*, could be detected by CAPS analysis. All of the cultivars of broccoli and cauliflower used in the CAPS analysis revealed the same band pattern to that

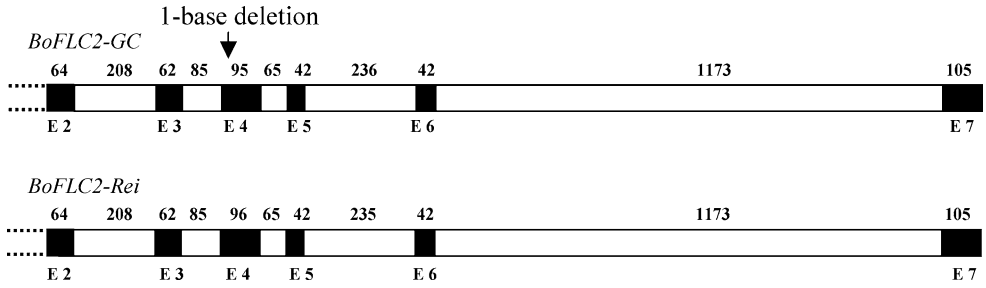

Fig. 2 Partial gene structure of *BoFLC2-GC* of 'Green Comet' and *BoFLC2-Rei* of Reiho DH lines. Exons (*E*) are represented by *filled boxes*, introns are represented by *open boxes*, and lengths in base pairs are given above. The *arrow* denotes the position of the 1-base deletion in exon 4 of *BoFLC2-GC*

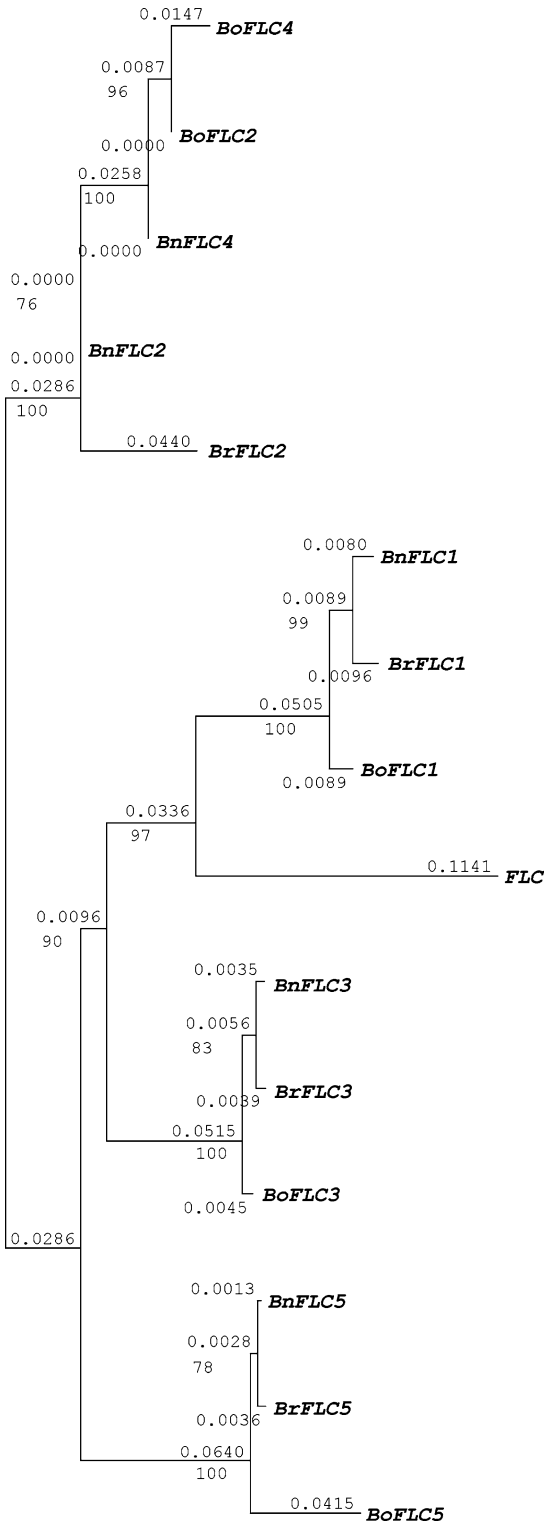

Fig. 3 Phylogenetic analysis of *Brassica FLC* homologs. Aligned coding sequences of *FLC* homologs from *B. napus* (*Bn*; Tadege et al. 2001), *B. rapa* (*Br*; Schranz et al. 2002) and *B. oleracea* (*Bo*) *BoFLC1*, *BoFLC3* and *BoFLC5* (Schranz et al., 2002) and *BoFLC4* (Lin et al., 2005) were used for phylogenetic analysis. Partial DNA sequences from exon 2 to exon 7 were analyzed using the neighbor-joining method. Genetic distance values are shown on the *top* of each branch, and bootstrap values are shown *below* the branches

of *BoFLC2-GC* (data not shown). Sequence analysis confirmed a 1-base deletion in this region of exon 4 of *BoFLC2* gene cloned from cauliflower. The cultivars of Kale and Brussels sprouts and cv. Neo Ruby of cabbage revealed the same band pattern to that of *BoFLC2-Rei* (data not shown).

Map construction and QTL analysis of flowering time

Out of the 83 primer pairs used in the analysis of CAPS, 65 generated clear PCR products. After restriction digestion of the PCR products, polymorphic bands were detected in products generated by 41 of the primer pairs (Table 2). In the RFLP analysis, 38 polymorphic loci were detected using 35 probes. Using SSR primers isolated from *B. rapa*, 14 polymorphic markers were detected. In the AFLP analysis, 36 polymorphic markers were detected in products generated from 20 primer pairs. In the SRAP analysis, 56 polymorphic markers were detected from 27 primer pairs. When a total of 225 polymorphic markers were analyzed, 187 markers were distributed in 12 linkage groups covering 540.3 cM, and the average interval between markers was 2.89 cM (Fig. 4). The assignment of the linkage groups to their respective chromosomes has tentatively been accomplished by alignment to the synteny maps of *B. napus* (Udall et al. 2005; Parkin et al. 2006) and *B. rapa* (Suwabe et al. 2006) and the integrated map of *B. oleracea* (Sebastian et al. 2000; http://www.grc.warwick.ac.uk/Content/sebastian_integ_map.htm). As a result, 4, 8, 3 and 4 common loci to the corresponding linkage groups were included in O2, O3, O5 and O9 linkage groups, respectively, and the remaining linkage groups had 1 or 2 common loci. Consistent linear orders among the markers were maintained between the base maps and our map.

The F_2 population obtained from the GC and Rei cross, which consisted of 134 plants, varied widely with respect to flowering time, containing early-, late- and non-flowering plants (Fig. 5). GC and F_1 plants flowered 55 and 72 days after transplantation, respectively, whereas Rei did not flower during the experiment.

The largest QTL effect (LOD 20.0) was significantly detected in the interval BRMS215 to F2-R4b on O2, based on the LOD threshold of 14.0 in the permutation test. In this region, *BoFLC2* was mapped. There was no QTL in the regions where the remaining *FLC* genes were mapped. The effects of the QTLs detected are listed in Table 3. The QTL in O2 explains about 36.8% of the phenotypic variance and is thought to play a major role in the control of flowering time. Small QTL effects, which were not significant in the permutation test, were detected in O2, O3, O6, O8 and O9. The

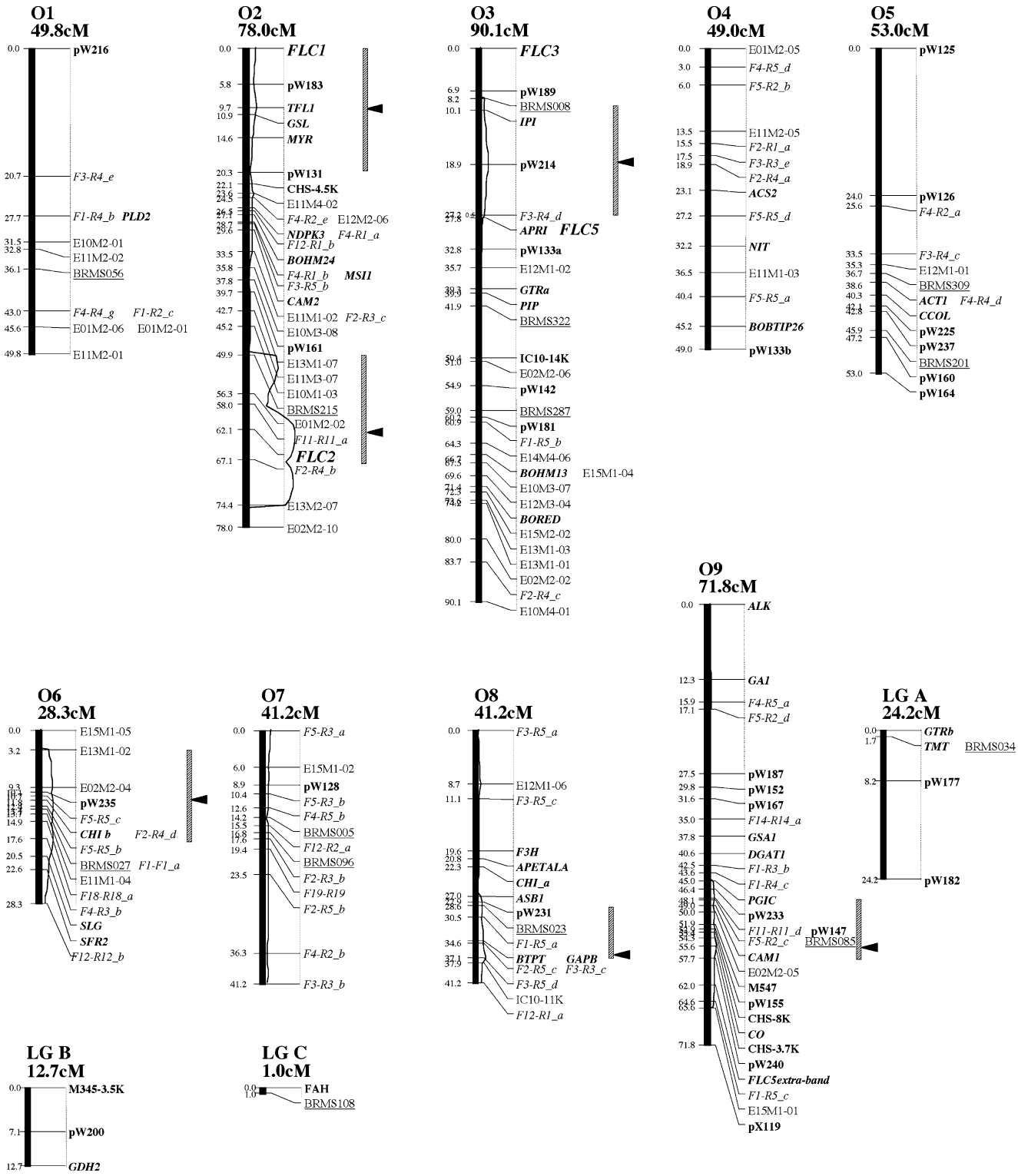

Fig. 4 Linkage map including RFLP, AFLP, SRAP, SSR and CAPS markers developed in a segregating F_2 population of broccoli × cabbage, and LOD profiles for loci controlling flowering time. The LOD threshold of 14.0 used to identify QTL is indicated by a *line*. *Box symbols* indicate the threshold of LOD >2.5. *Arrowheads* indicate peaks of the LOD value. *Numbers* to the left of each linkage group indicate the genetic distance from the top of each linkage group. Markers are denoted as follows. RFLP markers: *bold*, AFLP markers: *roman*, SRAP markers: *italic*; CAPS markers: *bold italic*, SSR markers: *underlined*

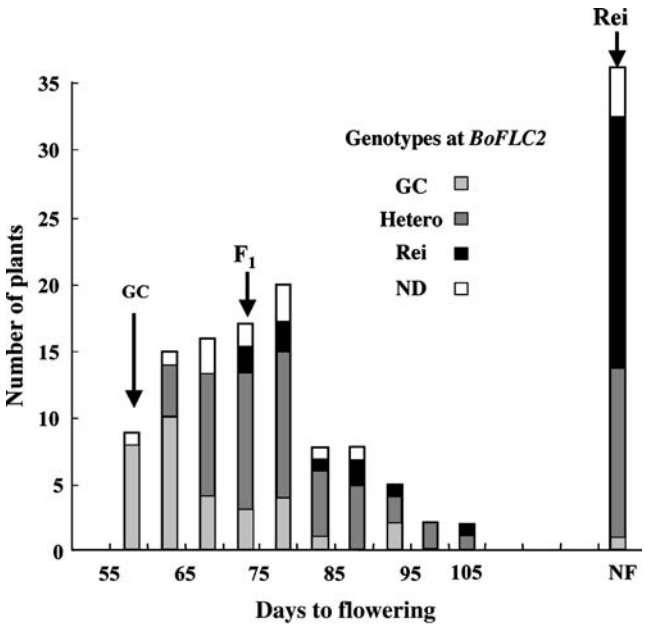

Fig. 5 Flowering time distribution in the *Brassica oleracea* (Green Comet × Reiho) F_2 population. Homozygotes of *BoFLC2-GC* (*GC*), homozygotes of *BoFLC2-Rei* (*Rei*), heterozygotes of *BoFLC2-GC*/*BoFLC2-Rei* (*Hetero*) and missing data (*ND*) are shown. Positions of the parental and F_1 plants in the distributions are shown. *NF* indicates plants that did not flower

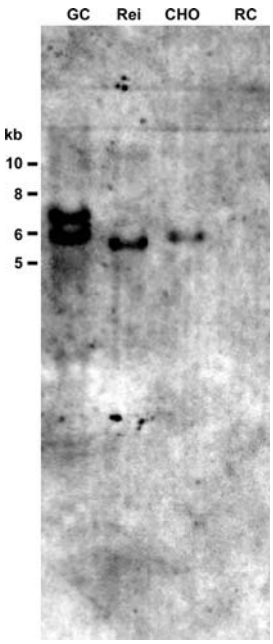

Fig. 6 Southern blot analysis of *Eco*RI-digested genomic DNA isolated from *B. oleracea* green comet (*GC*), Reiho (*Rei*), Choten (*CHO*) and the rapid-cycling line CRGC 3-1 (*RC*). The DNA blot was hybridized with a *BoFLC2* probe

early-flowering plants had the *BoFLC2-GC* genotype, whereas the late- and non-flowering plants had the *BoFLC2-Rei* genotype (Fig. 5).

Southern hybridization using *BoFLC* clones

Under the hybridization conditions used, each *BoFLC*-specific probe revealed specific bands in *B. oleracea* genomic DNA isolated from Rei, GC, Choten and CRGC. Using a *BoFLC2*-specific probe, no bands were detected in rapid-cycling CRGC, one band was detected in cabbage and two bands were detected in broccoli (Fig. 6). Hybridization using a *BoFLC5*-specific probe revealed one common band in broccoli, cabbage and CRGC (data not shown). With both *BoFLC1* and *BoFLC3* probes, broccoli and CRGC gave identical banding patterns and cabbage gave a different pattern (data not shown).

Expression of *FLC* mRNAs

To examine which *BoFLC* genes express in leaves and whether leaf age and plant age affect the expression of *BoFLC*mRNA, RT-PCR was performed using the *FLC*-specific primers. *BoFLC2* transcript of broccoli was detected in all samples analyzed; cotyledons, apexes and leaves, however, *BoFLC2* transcript level was very low in the leaves of 12-week-old plants and the cotyledon and the bands were very faint in agarose gel (Fig. 7), while visible in acrylamide gel (data not shown). Similar results were obtained in the expression of *BoFLC2* transcript of cabbage and cauliflower. Results of second leaf of 8-week-old plant in these plants were shown in Fig. 7. *BoFLC1* transcript was abundant in the apex of broccoli and decreased to a lesser extent in young and mature leaves of broccoli, but *BoFLC1* was expressed in the true leaves of cauliflower and cabbage. *BoFLC3* transcript was stably expressed in the cotyledon and the true leaves, and in the leaves obtained from aged plants.

No *BoFLC5* transcript was detectable in the apex, cotyledon and true leaves in cv. Shaster of broccoli and

Table 3 QTL analysis of flowering time in *B. oleracea*

Marker interval	Linkage group	Pesk LOD score	Additive effect[a] (days)	Variance explained (%)
BRMS215–F2-R4b	2	20.0	22.9	36.8
E13M1-02–F4-R3b	6	5.2	11.6	8.7
FLC1–pW131	2	3.5	7.7	4.3
BRMS023–F3-R5d	8	3.1	7.8	3.9
BRMS085–pW240	9	2.9	8.9	5.8
BRMS008–F3-R4d	3	2.6	−10	7.8

[a] Additive effect of biennial parent allele in days

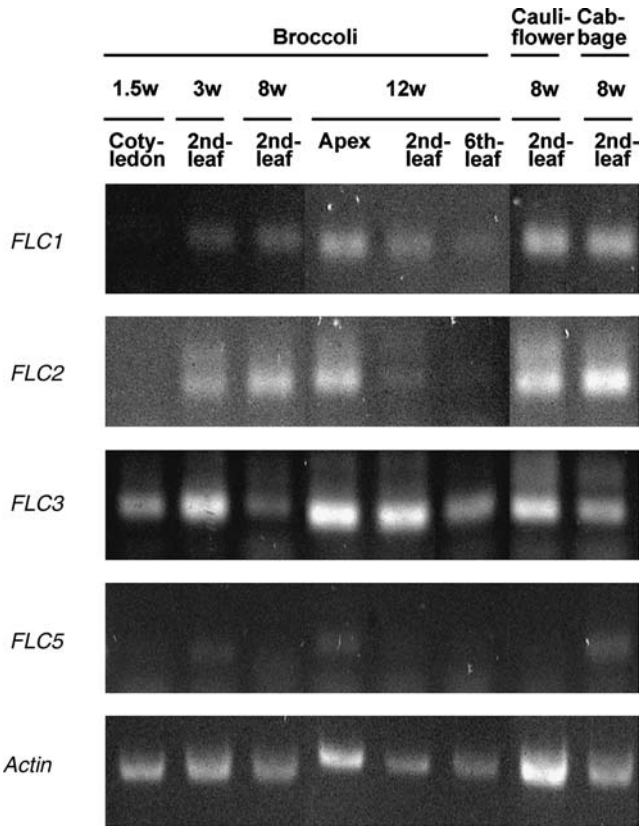

Fig. 7 Expression analysis of *BoFLC* genes. Expression levels of the four *BoFLC* genes in cotyledons, apex, 2nd leaves and 6th leaves obtained from 1.5- to 12-week-old broccoli and in 2nd leaves from 8-week-old cauliflower and cabbage were analyzed by RT-PCR. RT-PCR of *BoFLC1*, *BoFLC3* and *BoFLC5* was conducted using gene-specific forward primers designed to a highly variable region of exon 4 (Schranz et al. 2002) with a conserved exon 7 reverse primer (FLC-R). *BoFLC2* cDNA was amplified by using the sequence located in exon 3 (FLC2-F) with FLC-R. Actin gene was amplified as a control

cv. Snow Crown of cauliflower, whereas a small amount of *BoFLC5* transcript was detected in cv. Neo Ruby of cabbage.

Sequence analysis of the PCR products confirmed specific amplification using the *FLC*-specific primers (data not shown).

Discussion

FLC homologs in *B. oleracea*

B. rapa and *B. oleracea* have multiple *FLC* homologs, which were duplicated before *Brassica* species diverged from a *Brassica* ancestor (Schranz et al. 2002). Schranz et al. (2002) identified four *FLCs* (*BrFLC1*, *BrFLC2*, *BrFLC3* and *BrFLC5*) in *B. rapa* and three *FLCs* (*BoFLC1*, *BoFLC3* and *BoFLC5*) in *B. oleracea*; an *FLC2* homolog was not identified in *B. oleracea.* In the present study, we identified *BoFLC2* in *B. oleracea* that was included in the *FLC2* clade. The *BoFLC2* clone showed 91% homology to *BrFLC2* and also had 98% homology to *BoFLC4* (Lin et al. 2005). Lin et al. (2005) classified their *BoFLC4* clone using partial amino acid sequence and found homology to *BnFLC4* but not to members of the *FLC2* clade. Here we classified this *B. oleracea FLC* gene into the *FLC2* clade based on the nucleotide sequence in exons 2–7 and designated it *BoFLC2* to reflect its phylogenetic position. The difference may be due to the limited information available from partial amino acid sequence. Because *B. oleracea* and *B. rapa* each have four *FLC* genes, amphidiploid *B. napus* should have eight *FLC* genes. Tadege et al. (2001) identified only five *FLCs* in *B. napus* (*BnFLC1*, *BnFLC2*, *BnFLC3*, *BnFLC4* and *BnFLC5*), but recently Udall et al. (2005) reported the mapping of eight loci in *B. napus*, four in the *B. rapa* portion and four in the *B. oleracea* portion of the genome. It is interesting that rapid-cycling CRGC did not give any signal in the hybridization experiment using *BoFLC2* as a probe (Fig. 6). Schranz et al. (2002) were also unable to detect members of the *FLC2* clade in rapid-cycling *B. oleracea*. It is possible that some genotypes in *B. oleracea* are null in the *FLC2* allele because of the deletion of these genes. Alternatively, they may have genes that have large changes in their nucleotide sequences.

QTL analysis of flowering time

In cabbage, generally, to induce flowering approximately 8 weeks at 5°C after reaching a minimum stem diameter of 6 mm is required (Ito et al. 1966). For some cultivars of cabbage, vernalization requirements differed (Lin et al. 2005). Annuals such as broccoli and cauliflower usually do not require very low temperature for flowering (the non-vernalization type), whereas with some varieties it is know that after passing through the juvenile phase, low temperature (15°C) promotes curd formation in these ecotypes (Fujime 1988; Wurr and Fellows 2000). Although it is likely that there are optimal vernalization temperatures in *B. oleracea* ecotypes (cultivars), optimal vernalization temperatures are distinctly different between cabbage and broccoli. Thus, genetic analysis with the progeny of cabbage and broccoli can be informative for the appearance of vernalization.

Previous studies in crosses between annual and biennial plants disagree as to whether flowering habit of *B. oleracea* is a quantitative trait (Baggett and Wahlert 1975; Bagget and Kean 1989; Camargo and Osborn 1996) or a qualitative trait (Walkof 1963). In the present

study, the frequency distribution showed that the flowering time is controlled in a quantitative manner. F_1 plants represented annual types, which are late flowering, as compared with broccoli, suggesting that the annual habit is dominant over the biennial habit. These results are in agreement with those of Baggett and Wahlert (1975), Baggett and Kean (1989) and Camargo and Osborn (1996).

Vernalization is under control of different regulators in *Arabidopsis* (Amasino 2004). In *B. oleracea*, previous studies reported three QTLs in cross of cabbage and broccoli (Camargo and Osborn 1996) and three QTLs in O2, O3 and O9 in cross of rapid-cycling line and calabrese (Axelsson et al. 2001). Sebastian et al. (2002) identified two QTLs of flowering time in O7 and O8 in cross of cauliflower and Brussels sprout, and reported that the QTL in O8 can account for maintenance of vernalization. One of those QTLs corresponds to *CO* homologs mapped close to the flowering time QTLs (Lagercrantz et al. 1996; Bohuon et al. 1998; Axelsson et al. 2001). Similarly, we detected the small QTL effect in the position where *CO* was detected on O9. The other one small QTL was located in the interval from E13M1_02 to F4-R3b, adjacent to *SLG* locus, in O6. Linkage with the common loci of *SLG* and pW235 suggests that this QTL may be equivalent to that identified by Camargo and Osborn (1996), although there were differences in the magnitude and position of the effect. The remaining small QTLs detected in O3 and O8 are difficult to compare the previous reports due to a lack of common markers in the QTLs region, although our assignment of the linkage groups adopts standard nomenclature for the *Brassica* linkage group.

In *B. rapa*, two QTLs, *VFR1* and *VFR2*, were identified for vernalization-controlled flowering (Teutonico and Osborn 1995; Osborn et al. 1997). Kole et al. (2001) reported that an *FLC* co-segregated with *VFR2*. Furthermore, Schranz et al. (2002) mapped *BrFLC1* to *VFR2*. The above findings suggest that *BrFLC1* is responsible for vernalization-controlled flowering in *B. rapa*. *BrFLC2* and *BrFLC5*, however, were mapped to *FR1* and *FR2*, respectively. These two loci were previously reported to control flowering time that is not responsive to vernalization (Osborn et al. 1997), but Schranz et al. (2002) have shown that the variation in time to flowering caused by *FR1* (*BrFLC2*) and *FR2* (*BrFLC5*) is reduced by vernalization treatment. This suggests that *FR1* and *FR2* do indeed respond to vernalization treatment. In *B. rapa*, it is considered that *FLCs* act in a dosage-dependent manner to control the flowering response.

In the present study, the largest QTL was identified in O2 and explained about 36.8% of the phenotypic variation, indicating that this QTL mainly contributed to the control of flowering time in the F_2 progeny obtained from the cross of cabbage and broccoli. *BoFLC2* mapped to the peak of the flowering time QTL. Genotyping at the *BoFLC2* locus showed that most of the early flowering plants were homozygotes of *BoFLC-GC*, whereas most of the late- and non-flowering plants were homozygotes of *BoFLC-Rei* (Fig. 5). Non-flowering plants are thought to require low temperatures for flowering. The results may suggest that *BoFLC2* plays a role in flowering through vernalization. BRMS215 sequence, which mapped in the neighboring region of *BoFLC2*, has high homology to the sequence in the top of *Arabidopsis* chromosome 5 (data not shown). It is well known that *FLC* is located in the top of *Arabidopsis* chromosome 5. Therefore, the region of O2 containing *BoFLC2* is thought to correspond to the top of *Arabidopsis* chromosome 5. This is also supported from the *B. napus* synteny map where large portion of N2 and N12 correspond to *Arabidopsis* chromosome 5 (Parkin et al. 2006). The *Brassica* genomic region containing *BoFLC2* corresponds to the top of *Arabidopsis* chromosome 5. Among the flowering-related genes located in *Arabidopsis* chromosome 5 such as *FLC*, *CO*, *FY*, *LEY*, *EMF1* and *TFL1*, FLC is a key floral repressor in the maintenance of a vernalization response in *Arabidopsis* and mediates vernalization habit of many *Arabidopsis* ecotypes (Amasino 2004). Although we cannot exclude the possibility that the other candidate genes can harbor in the portion of the QTL containing *BoFLC2*, the results obtained from the QTL analysis and the genotyping of F_2 show that *BoFLC2* is a likely candidate for the largest QTL controlling flowering time.

Mutational change of genomic *BoFLC2*

In addition to deletion or a high divergence of *BoFLC2* allele in the rapid-cycling line CRGC 3–1, sequence analysis of *BoFLC2-GC* and *BoFLC2-cauliflower* revealed a frameshift (due to a 1-nucleotide deletion) in exon 4. The CAPS analysis using the polymorphic *Alu*I site in exon 4 detected this frameshift mutation in six cultivars and two strains of the annual type of *B. olearcea*, whereas there was no evidence of the frameshift in the portion of exon 4 in the biennial type of Kale, Brussels sprouts and cabbage.

Analysis of GC DNA by hybridization with a *BoFLC2*-specific probe revealed two bands. Sequence analysis showed no internal *Eco*RI restriction sites within these fragments, suggesting that the *BoFLC2* gene may have duplicated in GC. Analysis of PCR product containing the exon 4 of the *BoFLC2* amplified

from genomic DNA of GC showed that CAPS pattern using *Alu*I is consistent with that of the mutant containing the frameshift in the exon 4. Moreover, the frameshift in the exon 4 was confirmed in all the clones ($n = 8$) derived from the PCR product amplified from genomic DNA of GC (data not shown). Therefore, it is considered that *BoFLC2-GC* is homogeneous in the duplicated loci of GC. Overall, *FLC2* derived from the non-vernalization type accumulated mutational changes such as frameshifts, duplications or deletions.

Three diploid *Brassica* species, *B. oleracea*, *B. rapa* and *B. nigra* are thought to be derived from an ancestral species through whole genome triplication. Many of the genes in *Brassica* species are found in triplicated form. Since four clade members are found commonly in the *Brassica* species, *FLC* genes have duplicated in the triplicated ancestor genome before divergence into the three diploid *Brassica* species (Fig. 3). All of the four *FLC* members may be functional just after the duplication. Schranz et al. (2002) reported that *FLCs* act in a dosage-dependent manner to control the flowering response in *B. rapa.* The overexpression of *BnFLC* in *A. thaliana* (Tadege et al. 2001) provides evidence that multiple *FLC* loci encode functional gene products. However, the present study did not find QTLs in the region where *FLC* members other than *FLC2* were mapped.

Expression level of *BoFLC*

Using Northern hybridization analysis in non-vernalized leafy cabbage, Lin et al. (2005) reported that *BoFLC3* transcript was not detectable by a probe of 3′-untranslated region (UTR) of *BoFLC3*, whereas a large amount of *BoFLC4* transcript was detected by a 3′-UTR probe of *BoFLC4*. They also showed a decrease in the abundance of the *FLC* transcript after cold treatment, correlating with a breakdown in the vernalization requirement and concluded that *BoFLC4* (equivalent to our *BoFLC2-Rei*) mainly acts in vernalization-dependent flowering. In this study, there was no difference on the expression level of *BoFLC2* between broccoli and cabbage in RT-PCR, but a large QTL effect affecting flowering time was detected on O2 and the *BoFLC2* locus explained 36.8% of the phenotypic variation. This situation can be explained by a frameshift (due to a 1-nucleotide deletion) in exon 4 of *BoFLC2-GC*, indicating that *BoFLC2-GC* is not functional, even though the *BoFLC2* transcripts were expressed in annual broccoli. *BoFLC5* transcript level was extremely low, suggesting *BoFLC5* is not functional as a floral repressor. *BoFLC3* transcript was stably expressed in both young and mature leaves. It is possible that *BoFLC3* responds to vernalization in *B. oleracea*, but whether it is functional and a member of the vernalization cascade is still to be determined. QTLs for flowering were not found in the *FLC3* region in the present study or by Schranz et al. (2002), suggesting that there is no genetic difference in this position between parentages used in both studies.

The heterozygous plants (*BoFLC2-Rei*/*BoFLC2-GC*), which have functional FLC protein derived from the normal allele of the biennial plant, was expected to repress flowering under the warm climatic condition. However, all F_1 hybrid plants flowered and the flowering time of the hetrozygotes varied from early- to late-flowering and several heterozygotes did not flower in the end of experimental period (Fig. 5). It is commonly observed that F_1 hybrid plants obtained from cabbage and broccoli flower without vernalization (Camargo and Osborn 1996). One of the possible explanations is as follows. A single *FLC2* gene is not enough for the requirement of vernalization in some of the heterozygous plants (*BoFLC2-Rei*/*BoFLC2-GC*) and there could be modifying genes, some of which may be those determining the extent of vernalization. Recently, Sheldon et al. (2006), in their study of mRNA expression analysis of *FLC* and *SOC1* in *Arabidopsis*, reported that SOC1, a downstream target of FLC, is quantitatively induced by vernalization in a reciprocal manner to FLC. Genes located up- or downstream of *FLC* may act as modifiers in vernalization-mediated flowering.

Timing of flowering is under the control of different genes, which affect endogenous factors such as juvenile-, vernalization-, photoperiod phases, nutrient and plant age. The small five QTLs identified in the present study may help understand above various factors controlling flowering time. In addition, the results obtained from the polymorphism analysis of *BoFLC2* allele, the QTL analysis and the genotyping of F_2 suggested that *BoFLC2* is a likely candidate for the largest QTL controlling flowering time and *BoFLC2* plays some important roles in flowering through vernalization. Our present data, while very informative, do not offer the direct evidence to show the role of *BoFLC2* controlling vernalization. Thus, further studies are required to confirm the role of *BoFLC2* using a near-isogenic line with functional *BoFLC2* in the GC background as well as creating transgenic plants to do loss-of-function and/or gain-of-function experiments.

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