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Identification of quantitative trait loci controlling late bolting in Chinese cabbage (*Brassica rapa* L.) parental line Nou 6 gou

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To identify the genes responsible for varietal differences in bolting time in *Brassica rapa*, we constructed a linkage map of 220 simple sequence repeat (SSR) markers of *B. rapa*. To construct the map, we used F_2 progeny obtained from a cross between the late-bolting parental line Nou 6 gou (PL6) and the early-bolting parental line Nou 7 gou (A9709). The linkage map covered 875.6 cM and 10 major linkage groups. To investigate the nature of the vernalization response in the *B. rapa* parental lines, we examined bolting characteristics under two different conditions i.e., greenhouse and open field. Five quantitative trait loci (QTLs) that controlled bolting time were detected by experiments in greenhouse and open field. Interestingly, the QTLs identified in the greenhouse and field experiments did not map to the same loci. Three of five QTLs were colocalized with the orthologs of well-known flowering genes of *Arabidopsis*, including *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS T* (*FT*). The QTLs detected in this study may provide useful information for the selection of bolting traits in Chinese cabbage breeding.

Key Words: simple sequence repeat (SSR) markers, quantitative trait loci (QTL), bolting, *Brassica rapa*, *FLOWERING LOCUS C* (*FLC*), *FLOWERING LOCUS T* (*FT*).

Introduction

Vernalization is a process in which plants are exposed to low temperatures for efficient bolting, and this process occurs in many *Brassica* species. Vernalization is of two types, depending on the age of the plant, (1) seed vernalization, in which plants perceive the low temperature signal at the seed germination stage, and (2) plant vernalization, in which plants can perceive the low temperature signal only at a certain developmental stage. *B. rapa* is among the species that require seed vernalization, including important vegetables such as Chinese cabbages and turnip. Immature bolting, caused by low temperature conditions during spring, reduces yields and compromises the quality of Chinese cabbage. To prevent preharvest bolting, plastic tunnels have been used to maintain a sufficiently high temperature. Although some late-bolting varieties have been developed, unexpected bolting occurs in some cases. Therefore, it is necessary to identify DNA markers that are closely linked to late bolting.

FLOWERING LOCUS C (*FLC*), which encodes a MADS-box transcription factor, has been identified as a repressor of floral transition in *Arabidopsis thaliana* (Sheldon *et al.* 1999, 2000). *FLC* transcription is suppressed at a low temperature, and *FLC* downregulation promotes flowering (Michaels and Amasino 1999, Sheldon *et al.* 2000). *FLC* transcript levels determine the extent of the vernalization response in flower initiation in a dose-dependent manner (Sheldon *et al.* 2000). Genetic screening for mutants that retain late flowering after extended cold treatment identified two *Arabidopsis* genes, *VERNALIZATION 1* (*VRN1*) and *VERNALIZATION 2* (*VRN2*), that are involved in the vernalization response. *VRN1* encodes a DNA-binding protein and causes a stable repression of *FLC* (Levy *et al.* 2002). Vernalization itself epigenetically silences *FLC* by a process involving the polycomb protein VRN2. VRN2 promotes the trimethylation of histone H3K27 (Bastow *et al.* 2004, Gendall *et al.* 2001). *VERNALIZATION INSENSITIVE 3* (*VIN3*), which encodes a PHD-domain-containing protein, is involved in the initiation of *FLC* repression through alteration of the structure of *FLC* chromatin (Sung *et al.* 2006). In brief, *FLC* repression during vernalization is accomplished by the cooperative functions of these proteins.

FLOWERING LOCUS T (*FT*) is also involved in the floral transition pathway. The FT protein moves through the vascular tissues to the shoot apex and induces flowering (Abe *et al.* 2005, Kardailsky *et al.* 1999, Kobayashi *et al.* 1999). The FLC protein negatively regulates *FT* expression through a direct interaction with the first intron of *FT* (Helliwell *et al.* 2006, Lee *et al.* 2000, Samach *et al.* 2000).

The *Arabidopsis* and *Brassica* clades have been thought to diverge from a common ancestor, 17 to 18 million years ago (Yang *et al.* 1999, 2006). Because of chromosomal

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rearrangements, including fusions and/or fissions, *B. rapa* genome has become 4-fold larger than *A. thaliana* genome (Johnston *et al.* 2005), and the genome of *B. rapa* contains four copies of *FLC* and two copies of *FT* (Schranz *et al.* 2002, Yang *et al.* 2006).

Two quantitative trait loci (QTLs), *VFR1* and *VFR2*, that control days to flowering have been identified in *B. rapa* by non-vernalized experiments (Osborn *et al.* 1997). *VFR2* was found to contain *BrFLC1* (Schranz *et al.* 2002). Osborn *et al.* (1997) also reported two additional QTLs, *FR1* and *FR2*, which control vernalization-independent flowering. Schranz *et al.* (2002) mapped *BrFLC2* and *BrFLC5* to *FR1* and *FR2*, respectively. Li *et al.* (2009) reported that *BrFLC1* and *BrFLC2* were linked to QTLs that control bolting, budding, and flowering time. They used F_2 progeny derived from Yellow Sarson and the Japanese commercial variety "Osome." The natural variation of the splicing site in *BrFLC1* was thought to contribute to flowering time in a study using 121 *B. rapa* accessions (Yuan *et al.* 2009). Colocalization of the flowering-time QTL with flowering-related genes, including *BrFLC1* and *BrFLC2* has been detected in multiple segregating populations of *B. rapa* (Lou *et al.* 2007). These studies strongly suggest that the *BrFLC* genes have retained redundant functions in the vernalization response in *B. rapa*. In *B. napus*, three *FT* orthologs are associated with two QTLs for flowering time (Wang *et al.* 2009). Allelic variation in *FT* orthologs in *B. napus* is may be responsible for the variation in flowering time in winter and spring cultivars.

Although several studies on QTLs for flowering time in *B. rapa* are available, progenies of hybrids between distantly related lines have been used in these studies. In the breeding of Chinese cabbage cultivars, a cross between different Chinese cabbage lines is usually performed. For development of DNA markers useful in Chinese cabbage breeding, QTLs should be identified in progenies resulting from a cross between different Chinese cabbage lines. In this study, we used an extremely late-bolting parental line (PL6) and an early-bolting parental line (A9709) of Chinese cabbage, and performed QTL analysis to identify genetic markers associated with the late-bolting trait in PL6.

Materials and Methods

Plant materials and investigation of bolting time

Two parental lines of Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis*), Chukanbohon Nou 6 gou (PL6) and Chukanbohon Nou 7 gou (A9709), were used. An F_2 population (n = 478) was produced by self-pollinating an F_1 plant derived from a cross between PL6 (female) and A9709 (male). Temperature is the most important stimulus for bolting, and therefore, we divided the F_2 population into five subsets, and cultivated them under different conditions or during various periods (Table 1). One subset was cultivated in pots (diameter, 9 cm) in a heated greenhouse, and the other subsets were grown in the field at the National Institute of Vegetable and Tea Science from January to May in 2009 and 2010 (Tsu, Mie, Japan; 34°46′N, 136°25′E). In the pot-cultivation trials, the number of days required for the top of the inflorescence to reach 10 cm above the soil was recorded from the day of transfer to the greenhouse as the starting date. In the case of field cultivation, the length of the stem was recorded.

Linkage and QTL analysis

We constructed a linkage map by using genotyping data of 96 F_2 progeny derived from a cross between PL6 and A9709 in pot cultivation (09-pot population). The DNA markers used in the study were 43 simple sequence repeat (SSR) markers (prefix BRMS) reported by Suwabe *et al.* (2002, 2004, 2006), 30 SSR markers (prefixes Na, Ni, Ol and Ra) reported by Lowe *et al.* (2004), 99 SSR markers (prefix KBr) reported by Hatakeyama *et al.* (2010), 14 EST-based SSR markers (prefix BRE) developed by Abe *et al.* (unpublished data), and 7 SSR markers (prefix ENA or EJU) reported by Choi *et al.* (2007). For genotyping the *S* locus, we amplified *S locus glycoprotein* by using primer pairs reported by Nishio *et al.* (1996). To increase the number of SSR makers, we used the read2Marker program and developed 19 additional SSR markers (Fukuoka *et al.* 2005) from BAC sequences of the *B. rapa* KBr (H, B and S) libraries from the Brassica Genome Gateway (http://brassica.bbsrc.ac.uk/). The primers used for linkage map construction are listed in Supplemental Table 1. PCR reactions and detection of polymorphisms were performed as described previously (Hatakeyama *et al.* 2010). Linkage analysis was performed and genetic map was constructed using the Joinmap 4.0 software (van Ooijen 2006). The QTL analysis was performed using the MapQTL 5.0 software (van Ooijen 2004) under the "interval mapping" option. A second map was constructed using 96 F_2 plants in the field experiment that were transplanted in February 2009 (09-fieldA population).

Table 1. Five environmental conditions for bolting time evaluation

Trial	Sowing date	Date of transplanting	Date of recording	Conditions	Number of F_2 plants
09-pot	2008.11.13	5°C/24 h light for 49 days[a]		heated greenhouse[b]	96
09-fieldA	2009.1.8	2009.2.9	2009.4.23	Open field	96
09-fieldB	2009.1.27	2009.2.26	2009.5.12	Open field	96
10-fieldA	2010.1.8	2010.2.10	2010.4.28	Open field	109
10-fieldB	2010.2.16	2010.3.19	2010.5.27	Open field	81

[a] 25-days-old plants were treated with low temperature for 49 days under continuous light.

[b] The minimum temperarute in the greenhouse was 20°C.

The logarithm of odds (LOD) thresholds for QTLs ($\alpha = 0.05$) were estimated to be 3.5 and 4.5 in the 09-pot and 09-fieldA populations, respectively, by 1000 permutations.

RNA isolation and real-time RT-PCR analysis of BrFTa and BrFLC2

Two parental lines, A9709 and PL6, of 15-day-old plants were vernalized at 5°C for up to 49 days under a 12-h light cycle. Total RNA was extracted from leaf tissues at 7-day intervals for 49 days by using an RNeasy Plant Minikit (QIAGEN), according to the manufacturer's instructions. cDNA was synthesized using the PrimeScript™ RT reagent kit (TaKaRa) with random hexamer and oligo d(T) primers. Real-time RT-PCR was performed on a Thermal Cycler Dice Real-Time System (TaKaRa) using SYBR Premix Ex Taq II (TaKaRa). The real-time PCR primers for *BrFTa* and *BrFLC2* are listed in Supplemental Table 2. The transcript level of each gene was normalized to that of *BrACTIN* (Li *et al.* 2009).

Sequence and expression analysis of the BrFLC genes

To determine polymorphisms in *BrFLC1* and *BrFLC5*, genomic fragments were amplified using the specific primers listed in Supplemental Table 2 and a program consisting of 35 cycles (94°C for 30 s, 56°C for 30 s and 72°C for 2 min). PCR products were directly sequenced using an ABI3730 DNA Analyzer (Applied Biosystems) and analyzed using the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). For expression analysis of *BrFLC1*, young leaves were collected from 15-day-old plants in the greenhouse at 20°C, total RNA was extracted using an RNeasy Plant Minikit (QIAGEN), and RT-PCR was performed according to the method described by Yuan *et al.* (2009). To analyze the expression of 4 *BrFLC* genes during cold treatment, RT-PCR was performed using the specific primers listed in Supplemental Table 2 and a program consisting of 35 cycles (94°C for 30 s, 58°C for 30 s and 72°C for 30 s).

Results

Phenotypic variation

The bolting characteristics of the PL6 and A9709 parents, the F_1 hybrids, and the F_2 population were evaluated under five different environmental conditions (Table 1). To ensure that the plants in the pot experiment received an appropriate cold treatment, we incubated 25-day-old plants at 5°C for 49 days under continuous light. After the cold treatment, the plants were transplanted into pots (diameter, 9 cm) and placed in a heated greenhouse (February to March 2009). In the field experiment, seeds were germinated in pots placed in the greenhouse and transplanted into a field without subjecting them to a cold treatment. To examine bolting characteristics, we determined the number of days needed for the stem length to reach 10 cm (pot experiment) or the stem length per se (field experiment). The distribution of stem length and bolting time in the F_2 population is shown in Fig. 1. The bolting time significantly differed between A9709 and PL6, both in greenhouse and field cultivation (Fig. 1A, 1B). The F_2 populations did not show a typical discrete segregation, and this finding indicated that bolting is controlled by multiple genes. In the pot experiment (designated 09-pot), the number of days needed for the stem length to reach 10 cm was 10 ± 0, 27.5 ± 1.3 and 19.3 ± 1.0 (mean ± SD), in A9709, PL6, and F_1, respectively (Fig. 1A). In the field experiment in 2009 (designated 09-fieldA), the mean ± SD of the stem length was 33.7 ± 4.8, 5.7 ± 1.7 and 10.1 ± 1.7 cm in A9709, PL6, and F_1, respectively (Fig. 1B).

QTL for late bolting in PL6

QTL analysis in the 09-pot experiment identified three QTLs on two different linking groups (LGs), and that in the 09-fieldA experiment identified two QTLs on two LGs (Fig. 2). The QTL that had the greatest effect on bolting in pot cultivation, *pot-QTL1*, was detected on R07 located in close proximity to the KBrB092C03 marker and accounted for 25.3% of the observed variation (Table 2). The flowering

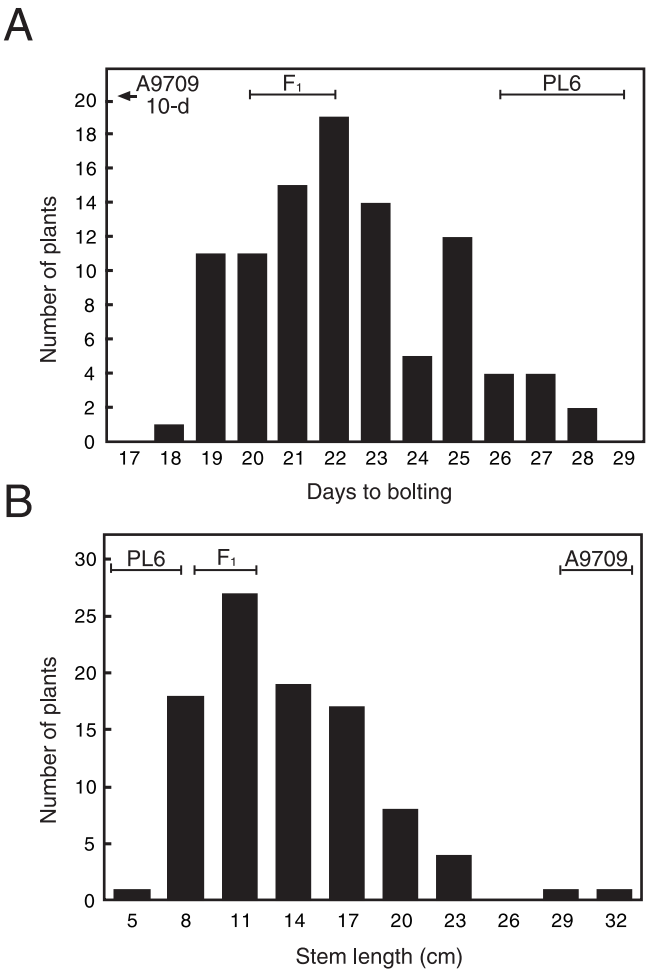


Fig. 1. Frequency distributions of days to bolting and stem length of 96 F_2 plants derived from a cross between the parental lines PL6 and A9709 in pot cultivation (A) and field cultivation (B).

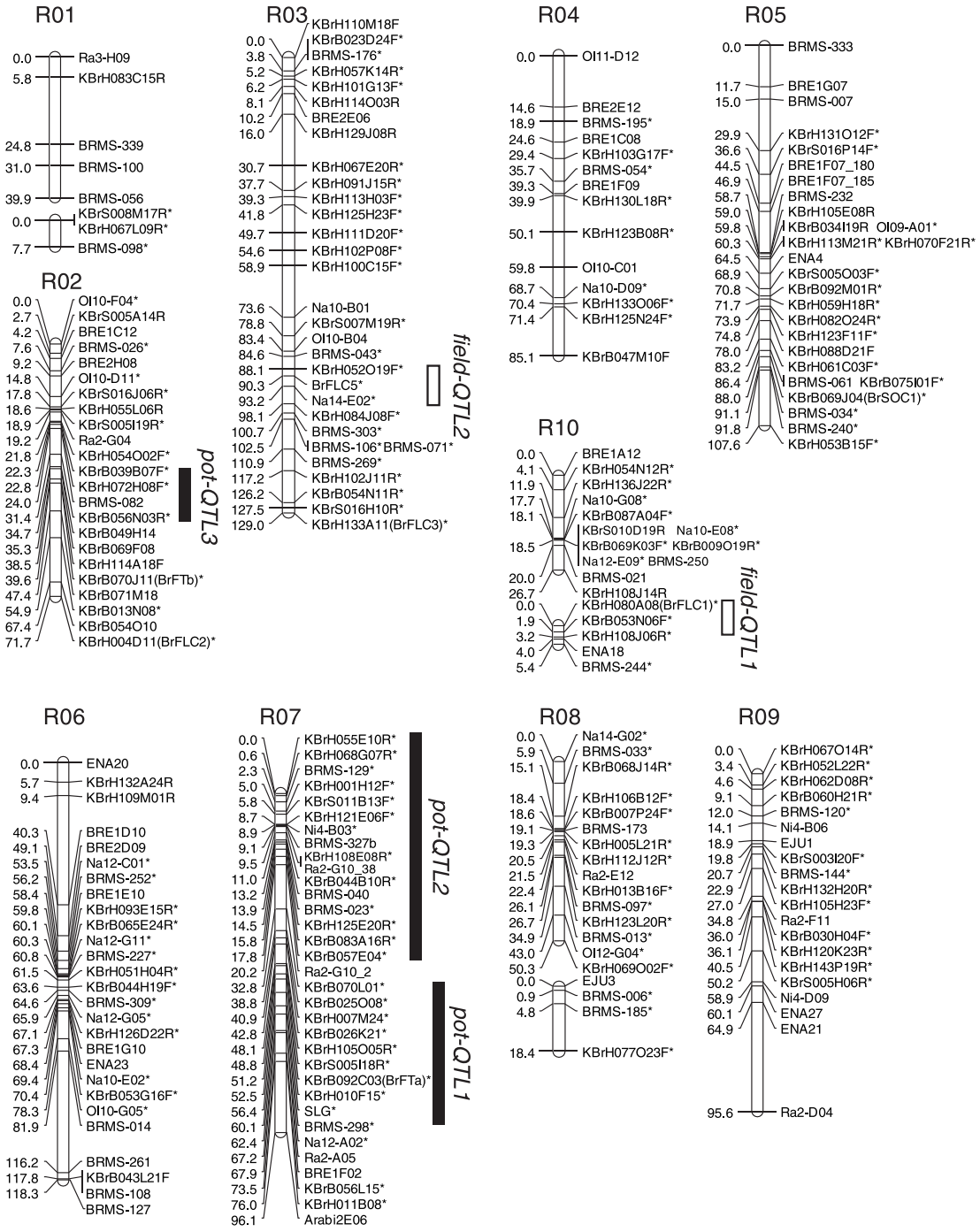


Fig. 2. Simple sequence repeat (SSR)-based linkage map constructed using genotyping data of the 96 F_2 plants derived from a cross between the parental lines A9709 and PL6 that were cultivated in a greenhouse in 2009. Linkage groups (LGs) are numbered according to the consensus LGs (R01 to R10) of the Multinational Brassica Genome Project (http://www.brassica.info/index.php), based on the common SSR markers. Marker names are indicated to the right of each LG. Markers with asterisks were used for the analysis of quantitative trait loci (QTLs) in the field experiment in 2009. Black and white boxes to the right of the LGs indicate QTLs detected in the greenhouse and field in 2009, respectively. The flowering-related genes, which are present in BAC sequences together with the mapped SSR markers, are shown in brackets.

promoter *BrFTa* was present in a BAC sequence containing KBrB092C03. Two other QTLs, *pot-QTL2* and *pot-QTL3*, were mapped on R07 and R02, respectively (Fig. 2). These three pot QTLs accounted for 64.2% of the observed variation (Table 2). No *FLC* or *FT* homolog was mapped near *pot-QTL2* and *pot-QTL3*. *Field-QTL1* and *field-QTL2* were located on R10 and R03, respectively, and accounted for 30.7% and 27.6% of the bolting variation, respectively (Fig. 2 and Table 2). *BrFLC1* and *BrFLC5* colocalized with *field-QTL1* and *field-QTL2*, respectively.

To examine the effects of the two genes, we classified the F_2 progeny on the basis of the marker genotype for each

Table 2. Results of QTL analysis for the late bolting of PL6

QTL	linkage group	Marker	candidate gene	09-pot experiment			09-fieldA experiment		
				LOD	Additive effect[a]	Variance explained (%)	LOD	Additive effect[b]	Variance explained (%)
pot-QTL1	R07	KBrB092C03(BrFTa)	*BrFTa*	6.09	−1.7	25.3			n.d.
pot-QTL2	R07	KBrH068G07R	n.d.	5.26	−1.7	22.3			n.d.
pot-QTL3	R02	KBrH072H08F	n.d.	4.54	−1.6	19.6			n.d.
field-QTL1	R10	KBrH080A08(BrFLC1)	*BrFLC1*		n.d.		7.64	3.8	30.7
field-QTL2	R03	BrFLC5	*BrFLC5*		n.d.		5.98	7.7	27.6

n.d., not-detected.

[a] Additive effect of A9709 allele in days to bolting.

[b] Additive effect A9709 allele in stem length.

Table 3. Relationship between two marker genotypes and the stem length (mean ± SD) in F_2 progeny

Marker genotype*		09-fieldA		09-fieldB		10-fieldA		10-fieldB	
KBrH080A08 (*BrFLC1*)	*BrFLC5*	No. of plants	stem length (cm)	No. of plants	stem length (cm)	No. of plants	stem length (cm)	No. of plants	stem length (cm)
AA	AA	5	21.2 ± 7.8^{a}	2	11.6 ± 5.3^{ab}	7	11.3 ± 1.7^{a}	4	11.3 ± 1.3^{a}
AA	AB	9	18.1 ± 1.8^{ab}	11	15.9 ± 5.8^{a}	21	9.5 ± 2.7^{a}	10	9.3 ± 1.8^{a}
AA	BB	4	11.9 ± 3.0^{bcd}	10	11.4 ± 2.6^{ab}	6	7.8 ± 0.8^{ab}	6	7.2 ± 2.3^{abc}
AB	AA	11	14.8 ± 3.8^{bc}	14	13.3 ± 4.5^{ab}	8	9.8 ± 4.7^{a}	5	8.2 ± 2.4^{ab}
AB	AB	21	12.0 ± 3.5^{cd}	25	11.1 ± 3.9^{b}	27	6.7 ± 1.7^{b}	24	7.4 ± 1.8^{bc}
AB	BB	22	9.0 ± 2.4^{d}	11	11.5 ± 3.6^{ab}	16	5.9 ± 1.5^{b}	9	5.9 ± 1.6^{bc}
BB	AA	4	14.0 ± 4.2^{bcd}	3	9.0 ± 1.6^{ab}	4	5.5 ± 1.3^{b}	5	7.2 ± 2.4^{abc}
BB	AB	16	9.6 ± 2.7^{d}	14	9.0 ± 4.0^{b}	13	5.9 ± 1.0^{b}	15	6.0 ± 1.9^{bc}
BB	BB	4	8.0 ± 1.8^{d}	6	9.3 ± 3.6^{b}	7	5.1 ± 0.7^{b}	3	4.3 ± 1.2^{c}
KBrB092C03 (*BrFTa*)	KBrH068G07R	No. of plants	stem length (cm)	No. of plants	stem length (cm)	No. of plants	stem length (cm)	No. of plants	stem length (cm)
AA	AA	10	13.8 ± 6.8^{ab}	7	16.3 ± 2.6^{a}	3	11.7 ± 8.1^{a}	12	9.3 ± 1.8^{a}
AA	AB	11	13.0 ± 6.8^{ab}	12	12.6 ± 5.1^{ab}	16	7.9 ± 2.5^{ab}	9	7.0 ± 2.3^{ab}
AA	BB	3	9.0 ± 1.7^{ab}	5	10.5 ± 6.3^{ab}	5	7.2 ± 1.8^{ab}	2	7.0 ± 1.4^{ab}
AB	AA	11	13.4 ± 3.8^{ab}	11	13.8 ± 5.3^{ab}	15	7.9 ± 2.7^{ab}	3	8.7 ± 2.1^{ab}
AB	AB	27	13.8 ± 4.5^{a}	29	11.1 ± 4.0^{ab}	31	7.4 ± 2.8^{ab}	21	8.1 ± 1.9^{ab}
AB	BB	8	10.9 ± 4.2^{ab}	18	10.5 ± 3.1^{ab}	8	6.6 ± 2.4^{ab}	10	5.9 ± 1.8^{b}
BB	AA	4	11.6 ± 3.4^{ab}	2	12.0 ± 0.9^{ab}	3	6.7 ± 1.2^{ab}	3	7.3 ± 3.1^{ab}
BB	AB	10	11.4 ± 2.8^{ab}	7	10.2 ± 3.9^{ab}	17	7.7 ± 2.3^{ab}	8	6.3 ± 1.5^{ab}
BB	BB	10	7.8 ± 1.6^{b}	4	7.0 ± 1.3^{b}	8	5.8 ± 1.7^{b}	13	5.8 ± 2.6^{b}
A9709 (AA)		3	33.7 ± 4.8	3	Flowered	3	48.5 ± 21.5	2	37.0
PL6 (BB)		3	5.7 ± 1.7	0	–	3	4.5 ± 0.9	3	4.7 ± 0.6

* AA, genotype of A9709 (early-bolting); BB, genotype of PL6 (late-bolting).

Values followed by the same letter are not significantly different at the 5% level, as determined by Tukey-Kramer HSD test.

gene and correlated the stem length with the 9 different genotypes (Table 3). The F_2 progeny with the *BrFLC1* and *BrFLC5* alleles derived from the PL6 parent showed significant delays in stem elongation, except in the 09-fieldB experiment (Table 3). In the 09-fieldB experiment, *BrFLC1* appeared to predominantly determine late bolting whereas *BrFLC5* had no effect. Because *BrFLC1* and *BrFLC5* played important roles in determining the timing of bolting in the field experiments, we next investigated the effects of other genetic markers, namely *BrFTa* and KBrH068G07R, which affected the timing of bolting in the pot experiment (Fig. 2). Although we failed to detect any QTLs for these two markers in the field experiments, we observed a significant selection effect (Table 3). For example, in three of the four field experiments (09-fieldB, 10-fieldA and 10-fieldB), the F_2 progeny with the *BrFTa* and KBrH068G07R alleles from PL6 showed significantly shorter stem than did the F_2 progeny with the *BrFTa* and KBrH068G07R alleles from A9709 (Table 3).

Expression analysis of BrFTa and BrFLC2

Several studies have shown that the QTL that controls flowering time is located near *BrFLC2* in *B. rapa* (Li *et al.* 2009, Lou *et al.* 2007, Osborn *et al.* 1997). Similarly,

Okazaki *et al.* (2007) reported that *BoFLC2* plays a central role in the repression of flowering in *B. oleracea* (Okazaki *et al.* 2007). Wang *et al.* (2009) reported that *BnC6FTa*, the orthologous gene of *BrFTa*, was associated with the major QTL for flowering time in *B. napus*. Furthermore, our QTL analysis showed that *BrFTa* was located near QTL for bolting time in *B. rapa* (Fig. 2). Next, we quantified the expression of *BrFTa* and *BrFLC2* in the pot experiment at 7-day intervals for 49 days by real-time RT-PCR. The expression of *BrFTa* increased gradually in A9709, whereas its expression was stable in PL6 (Fig. 3A). To investigate the effect of *BrFLC2* on bolting after vernalization under artificial conditions, we monitored the expression of *BrFLC2* during vernalization (Fig. 3B). However, we were unable to detect any significant differences in *BrFLC2* expression between PL6 and A9709 under these conditions. In addition to *BrFLC2* expression analysis, no peaks of LOD scores were observed in nearby regions of *BrFLC2* in the QTL analysis. These results suggest that late bolting in PL6 under conditions of continuous exposure to low temperatures is primarily determined by the repression of *BrFTa* during vernalization and is independent of *BrFLC2*.

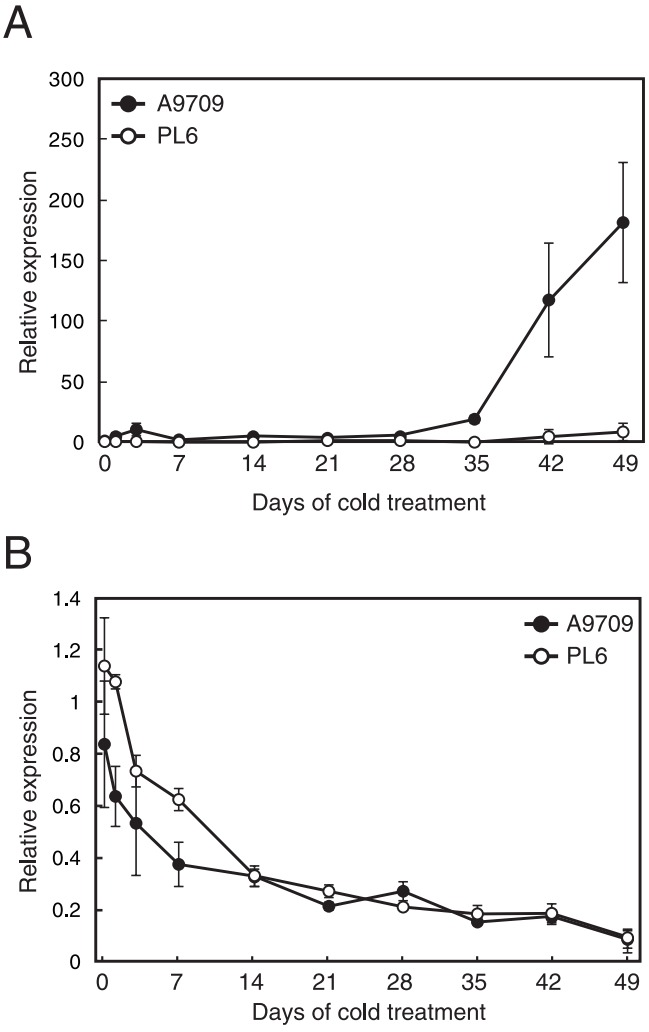


Fig. 3. Expression analysis of *BrFTa* and *BrFLC2* in response to vernalization. Two parental lines (A9709; closed circles and PL6; open circles) of 15-day-old plants were vernalized at 5°C for up to 49 days under a 12-h light cycle. The *BrFTa* (A) and *BrFLC2* (B) transcripts were examined in young leaves at the end of the light period. Transcript levels were analyzed by real-time RT-PCR and normalized to the levels of *BrACTIN* (Li *et al.* 2009). Error bars represent standard deviations of the mean.

Genomic structure and expression of BrFLC genes

To elucidate the genome structure of *BrFLC1* and *BrFLC5*, we determined the nucleotide sequences from the 5′-UTR to exon 7 of *BrFLC1* and from exon 4 to exon 7 of *BrFLC5*, respectively (Fig. 4A). A sequence comparison of the *BrFLC1* alleles from A9709 and PL6 identified a single nucleotide polymorphism in exon 2. In *BrFLC5*, a 17-bp deletion was identified in intron 6 of the A9709 allele. To investigate the effect of these nucleotide polymorphisms on *BrFLC1* expression, we performed RT-PCR for 35 cycles with primers in the 5′-UTR and exon 7. Surprisingly, a radical reduction in the *BrFLC1* transcript level was observed in A9709, and an alternatively spliced transcript was identified (Fig. 4B). According to the existing literature, alternative splice variants generated by a G-to-A polymorphism at the 5′ splice site in intron 6 of *BrFLC1* are associated with a flowering phenotype (Yuan *et al.* 2009). However, although one SNP site was detected at exon 2, no differences were detected in intron 6 between A9709 and PL6 according to genomic sequencing. Next, we cloned and sequenced this alternative transcript derived from A9709. In the longer transcript, intron 3, consisting of 77 nucleotides, was retained in the mature mRNA. This resulted in a frame-shift mutation and introduced a stop codon, giving rise to a truncated protein of 137 amino acids (Fig. 4C). To investigate the response of 4 *BrFLC* paralogs under cold conditions, we performed RT-PCR analysis using specific primer pairs that amplify exon 4 to exon 7 of each *BrFLC* gene (Fig. 4D). During the cold treatment period, the expression of the four *BrFLC* genes was decreased. However, the expression levels of *BrFLC1* and *BrFLC5* were higher in PL6 than in A9709 during the cold treatment, and the expression was slightly detected even after seven weeks of cold treatment. These results suggest that the late bolting of PL6 was controlled by *BrFLC1* and *BrFLC5*.

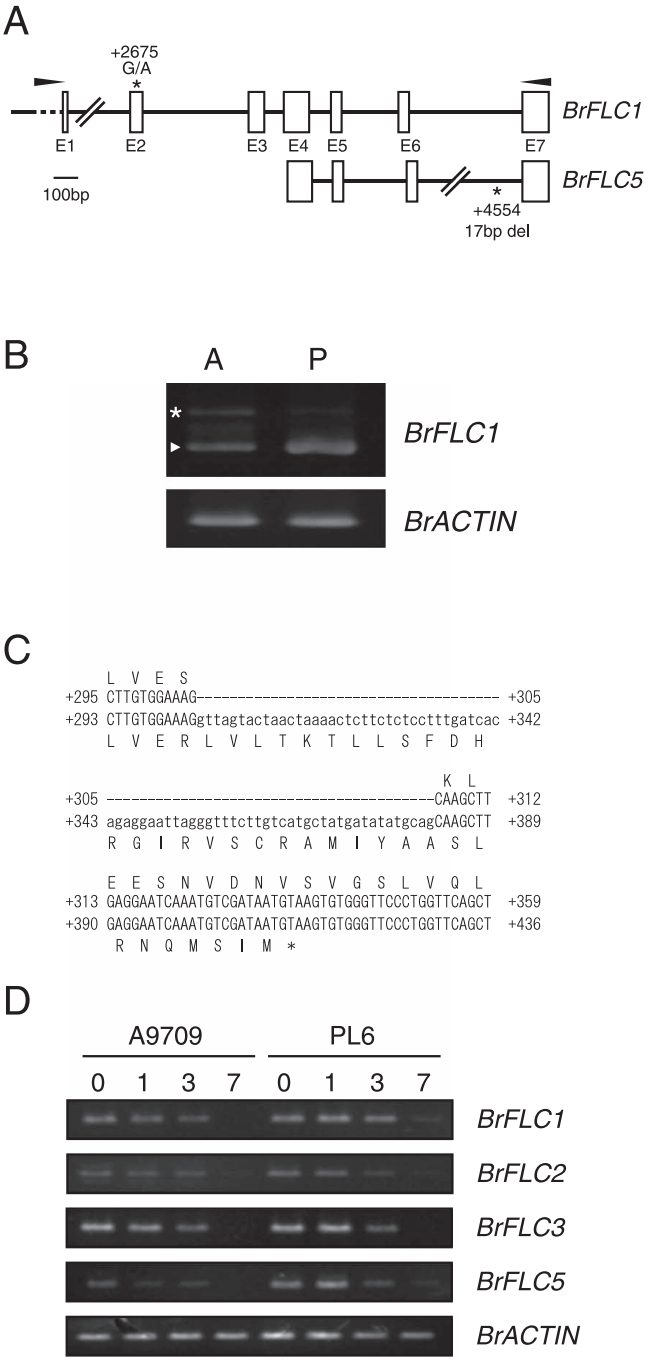
Discussion

The timing of bolting and flowering is regulated by endogenous factors and environmental stimuli. Many genes controlling this pathway have been isolated and characterized in *Arabidopsis* (Crevillen and Dean 2010, Dennis and Peacock 2007). To obtain information regarding the marker-assisted selection of *Brassica* crops, we mapped vernalization genes and flowering-related genes on a genetic map constructed using an F_2 population derived from two parental lines that show clearly different bolting times. Our data suggest that

known genes involved in the vernalization pathway in *Arabidopsis* can be mapped at QTL positions. Unexpectedly, we were unable to detect any common QTLs for different environmental conditions, i.e., greenhouse and open field.

Alternative transcripts of BrFLC1

In this study, we showed that *BrFLC1* was colocalized with the *field-QTL1*, and its allele from PL6 delayed bolting. Although we identified a nucleotide substitution at 2675 bp in exon 2 of *BrFLC1* (causing a nonsynonymous change (Q69R) in the PL6 allele), this did not directly explain the difference in bolting times between PL6 and A9709. Interestingly, we detected an alternative *BrFLC1* transcript in A9709 (Fig. 4B). This alternative transcript retains intron 3 and gives rise to a truncated protein (Fig. 4C). Yuan *et al.* (2009) reported that a substitution at the 5′ splice site in intron 6 of *BrFLC1* causes alternative splicing and affects flowering time. Alternative splicing can result in the generation of protein with new functions through exon sliding (Tarrio *et al.* 2008). However, it often creates transcripts with premature stop codons, which are generally degraded by nonsense-mediated decay (Barbazuk *et al.* 2008). Although it is unclear whether splicing is influenced by SNPs in exon 2 of *BrFLC1*, the possibility of an SNP in the exon causing the alternative splicing has been suggested in wheat (Sun *et al.* 2010).



Differences in bolting between pot and field experiments

We identified three QTLs in pot experiments (*pot-QTL1*, *pot-QTL2* and *pot-QTL3*) and two in field experiments (*field-QTL1* and *field-QTL2*). Three of the five QTLs colocalized with homologs of well-known flowering-related genes from *Arabidopsis*, such as *FT* and *FLC* (Fig. 2). Unexpectedly, no QTLs involved in bolting variation were detected in both the pot and field experiments. Several studies involving QTL analyses have suggested that *Brassica* possesses four *FLC* orthologs that control the vernalization response (Li *et al.* 2009, Osborn *et al.* 1997, Schranz *et al.* 2002, Yuan *et al.* 2009). However, in the pot experiment, no major QTL was detected in any of the four *FLC* orthologs. Real-time RT-PCR results indicated that one of the *FLC* genes in *Brassica*, *BrFLC2*, showed similar expression patterns in A9709 and PL6. In contrast, *BrFTa* was dramatically induced after 35-day cold treatment in only A9709 (Fig. 3A).

In the pot experiment, the temperature was maintained at

Fig. 4. Nucleotide polymorphisms and gene expression analysis of *BrFLC* genes. (A) DNA sequences of *BrFLC1* and *BrFLC5* from exon 1 to exon 7 and from exon 4 to exon 7, respectively, were determined. Exons, introns, and the 5′-UTR are shown as open boxes, black bars, and a dotted line, respectively. The position of the translational start site is indicated as +1. Polymorphisms between A9709 and PL6 are indicated with asterisks. (B) RT-PCR amplification of *BrFLC1*. Leaf samples were collected from 15-day-old plants. Primer annealing sites are indicated in (A) by black triangles located at the 5′-UTR and exon 7 of *BrFLC1* (Yuan *et al.* 2009). The alternative and normal transcripts in A9709 are indicated by an asterisk and a white triangle, respectively. *BrACTIN* was amplified as an internal control. A: A9709; P: PL6. (C) Nucleotide and deduced amino acid sequences of the region giving rise to alternative splicing (exon 3 to exon 4) of *BrFLC1* in A9709. The normal transcript (PL6) is shown in the upper lane, and the alternative transcript (A9709) is shown in the lower lane. The nucleotide sequence of intron 3 is shown in lower case. The position of the translational start site in the *BrFLC1* cDNA is indicated as +1. The premature stop codon (TAA) is indicated with an asterisk. (D) Expression of *BrFLC* genes during cold treatment, harvested after 0, 1, 3, or 7 weeks of growth at 5°C, as indicated in above the figure. *BrACTIN* was amplified as an internal control.

5°C for 49 days during the cold treatment. In the field experiment, the temperature showed daily fluctuations during the winter. Although the expression of *VIN3*, which is the activator of *FLC* repression, is upregulated by cold treatment for 20 days, its expression is repressed once the plant is again exposed to normal temperatures (Sung and Amasino 2004). To accomplish robust repression of *FLC*, *Arabidopsis* requires continuous cold treatment for at least 3 weeks (Gendall *et al.* 2001). Probably because a temperature sufficiently low for *FLC* repression was achieved in the pot experiment, QTLs were not detected near *FLC* but were detected near flowering-promoting genes such as *FT* (Fig. 2 and Table 2). In the field experiments, which involved temperature oscillations and a gradual temperature decrease, we detected QTLs for genes involved in the vernalization pathway (Fig. 2 and Table 2).

Although the QTLs at *BrFTa* and KBrH068G07R were detected only in the pot experiment, their effects on the selection process were also observed in the field experiment (Table 3). This suggests the existence of minor QTLs with a slight but distinctive effect on the differences in bolting between PL6 and A9709. In fact, we did not isolate any F_2 progeny that bolted as early as A9709 in any of the experiments. This indicates that many loci are involved in bolting in *B. rapa*, some of which remained undetected in this study.

In summary, we identified three QTLs at the periphery of orthologous genes of well-known flowering genes from *Arabidopsis*, including *FLC* and *FT*. We expect the *BrFLC* genes to be mainly involved in the low-temperature requirement, whereas *BrFTa* and KBrH068G07R affect elongation of the stem after exposure to low temperature. We used two different environments to identify four genetic markers that almost completely explain the late bolting feature of PL6. Marker-assisted selection by combining these four markers will enable the selection of a late-bolting Chinese cabbage.

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