RESEARCH PAPER

A naturally occurring splicing site mutation in the *Brassica rapa* FLC1 gene is associated with variation in flowering time

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Abstract

FLOWERING LOCUS C (FLC), encoding a MADS-domain transcription factor in *Arabidopsis*, is a repressor of flowering involved in the vernalization pathway. This provides a good reference for *Brassica* species. Genomes of *Brassica* species contain several FLC homologues and several of these colocalize with flowering-time QTL. Here the analysis of sequence variation of *BrFLC1* in *Brassica rapa* and its association with the flowering-time phenotype is reported. The analysis revealed that a G → A polymorphism at the 5′ splice site in intron 6 of *BrFLC1* is associated with flowering phenotype. Three *BrFLC1* alleles with alternative splicing patterns, including two with different parts of intron 6 retained and one with the entire exon 6 excluded from the transcript, were identified in addition to alleles with normal splicing. It was inferred that aberrant splicing of the pre-mRNA leads to loss-of-function of *BrFLC1*. A CAPS marker was developed for this locus to distinguish Pi6+1(G) and Pi6+1(A). The polymorphism detected with this marker was significantly associated with flowering time in a collection of 121 *B. rapa* accessions and in a segregating Chinese cabbage doubled-haploid population. These findings suggest that a naturally occurring splicing mutation in the *BrFLC1* gene contributes greatly to flowering-time variation in *B. rapa*.

Key words: *BrFLC1*, flowering time, splicing pattern, splicing site mutation.

Introduction

Flowering is one of the most important developmental traits for the production of *Brassica rapa* crops, including various vegetable crops such as Chinese cabbage (ssp. *pekinesis*), pak choi (ssp. *chinensis*), wutacai (ssp. *narinosus*), turnip (ssp. *rapa*), caixin (ssp. *parachinensis*), mizuna (ssp. *nipposinica*), and broccolito (ssp. *broccolito*), as well as oilseed crops such as turnip rape (ssp. *oleifera*) and yellow sarson (ssp. *tricolaris*) (Gómez-Campo, 1999). Premature flowering triggered by low temperature leads to a reduction in the yield and quality of the harvested products of *B. rapa*. Therefore, understanding the mechanism of flowering control is important in agronomic practice in preventing *B. rapa* from flowering prematurely.

The timing of flowering is regulated by several factors, including endogenous cues and environmental stimuli. Many genes involved in the regulation of flowering time have been identified from *Arabidopsis* (Simpson et al., 1999; Boss et al., 2004). In *Arabidopsis*, the flowering-time genes function on four major promotion pathways: photoperiod, vernalization, autonomous, and gibberellin (Simpson et al., 1999; Mouradov et al., 2002; Jack, 2004; Alexandre and Hennig, 2008). FLOWERING LOCUS C (FLC), involved in the convergence of autonomous and vernalization pathways, 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).
et al. (2008; De Lucia et al., 2008). Both vernalization and autonomous pathways repress the expression of FLC and promote flowering in vernalization-responsive late-flowering plants (Lee and Amasino, 1995; Sheldon et al., 2000; Sung and Amasino, 2006; Schmitz and Amasino, 2007). Several genes involved in vernalization, including VIN3, VRN1, and VRN2 (Sung and Amasino, 2005), and genes in the autonomous pathway, including FCA, FLD, FVE, FPA, LD, and FLK, repress FLC expression (He and Amasino, 2005). However, the genes FRI, FRL1, FRL2, VIP3, VIP4, ELF7, ELF8, EF5, and PIE1 are positive regulators of FLC (Rouse et al., 2002).

Some Brassica species, including B. rapa, B. oleracea, and B. napus, are typical vernalization-sensitive plants. Genes homologous to Arabidopsis FLC play major roles in the vernalization response in Brassica species (Osborn et al., 1997). In B. rapa, several QTL (VFRI, -2, and -3 and FRI, -2, and -3) for flowering time were identified in an F2 and a recombinant inbred line population derived from a cross between an annual and a biennial oil type (Teutonico and Osborn, 1994; Osborn et al., 1997; Schranz et al., 2002; Lou et al., 2007). VFR2 was estimated to have a large effect on flowering time, was responsive to vernalization, and was suggested to be homologous to FLC of Arabidopsis (Kole et al., 2001). A further study confirmed that VFR2 locates at the BrFLC1 locus, FRI at the position of BrFLC2, and FRI at BrFLC5 (Schranz et al., 2002). Recent studies revealed that four B. rapa flowering-time genes BrFLC1, BrFLC2, BrFLC3, and BrFLC5 were assigned to linkage groups A10, A02, A03, and A03, respectively (Kole et al., 2001; Schranz et al., 2002; Kim et al., 2006; Yang et al., 2006). The QTL with BrFLC2 as candidate gene on A02 was identified in different populations and environments/locations (Lou et al., 2007). Overexpression of BrFLC1, BrFLC2, and BrFLC3, isolated from Chinese cabbage cv. Chifu, in Arabidopsis, and BrFLC3 in transgenic Chinese cabbage delayed flowering time (Kim et al., 2007). Five FLC-related homologues (BrFLC1–5) isolated from B. napus delayed flowering significantly when they were expressed in Arabidopsis (Tadege et al., 2001). There are four FLC copies in B. oleracea (Schranz et al., 2002; Lin et al., 2005; Okazaki et al., 2007), but only BoFLC2 was found as a putative candidate gene for a large effect QTL for flowering time in an F2 population from a cross of a nonvernalization-type broccoli and a vernalization-type cabbage (Okazaki et al., 2007). All these studies indicate that FLC homologues in Brassica species act similarly to AtFLC and play a central role as repressors of flowering. However, by contrast, a recent study on sequence polymorphism of FLC paralogues in B. oleracea indicated that BoFLCs do not constitute strong candidate genes for flowering-time QTL in the backcross population studied, although four BoFLCs were located to their respective linkage groups (Razi et al., 2008). It is expected that finding FLC genes as candidates will depend on the parental lines used in different experiments.

As in other organisms, plant genes contain conserved 5′ splice sites (exon/intron junction AG/GTAAG) and 3′ splice sites (intron/exon junction TGCAG/G). The first two nucleotides in the 5′ splice site intron junction sequence, +1G and +2T, have shown 100% and 99% conservation, respectively, among over 1000 Arabidopsis introns studied (Brown, 1996; Lorkovic et al., 2000). Mutations in splice sites can abolish splicing or lead to exon skipping, i.e. the affected exon and both flanking introns are removed in a single splicing event (Simpson et al., 1998; Lorkovic et al., 2000). The mutations in splice sites could also block splicing at the normal splice site or lead to intron retention, i.e. cryptic splice sites at different positions are activated, and cryptic splicing of the affected exons together with the downstream intron are retained (McCullough et al., 1993).

Loss-of-function alleles in FLC resulting in alternative splicing were found in Arabidopsis. A normal-length FLC transcript with nonsense mutation as well as an alternatively spliced transcript lacking exon 6 were found in the early flowering Van-0 accessions and an alternatively spliced FLC allele that behaves as a null allele was also identified in Bur-0 with a vernalization-independent, late-flowering habit (Werner et al., 2005). Three natural FLC alleles with severely affected protein function in Cen-0, LI-2, and Cal-0 accessions were discovered, and at least one of the very early accessions, LI-2, likely carries a null allele (Lempe et al., 2005). These findings demonstrate that FLC alleles with severely compromised protein function in Arabidopsis can result from alternative splicing. Schranz et al. (2002) identified alternate splice variants of BrFLC5 in a biennial oilseed cultivar, but there is no further report on the relationship between the alternative splicing and the flowering-time phenotype.

Flowering time is one of the most important agronomic traits and a wide range of flowering-time variation exists among natural B. rapa accessions, which provides an excellent resource for dissecting the molecular basis of flowering-time control. In B. rapa, although four BrFLCs have been identified (Schranz et al., 2002), it has not been clarified how differences in the alleles of the FLC genes contribute to the variation in flowering time.

Here, a naturally occurring splicing site mutation in the BrFLC1 gene is reported. A mutation of G→A at the splicing site of BrFLC1, resulting in three alternative splicing patterns, was identified. The association of the splicing mutation with flowering-time variation was confirmed by applying a cleaved amplified polymorphic sequence (CAPS) marker recognizing the G→A mutation at the splicing site.

Materials and methods

Plant materials

To characterize the natural variation of flowering time in Brassica rapa, a total of 121 accessions belonging to 11 cultivar groups was screened. The collection includes 100 doubled-haploid (DH) lines derived from several commercial hybrids, six commercial hybrids, and 15 inbred lines (Table 1). A selected subset of 30 accessions (Table 2) with a wide range of flowering-time variation was used for BrFLC1 sequencing.
To verify the involvement of *BrFLC1* in flowering-time variation, 180 Chinese cabbage DH lines from the Y177×Y195 population (designated BrIVFhn) were analysed as described by Wu et al. (2008). BrIVFhn is derived from a cross between female parent Y177, a DH line derived from a late flowering winter-type Japanese cultivar named ‘Jianchun’ and male parent Y195, a DH line derived from an early-flowering Chinese summer type cultivar named ‘Xiayang’. Both parental DH lines were included in the collection of 121 *B. rapa* accessions.

### Growth conditions

To investigate the flowering-time variation, plants were grown in the open field and in a growth chamber. The evaluation of the 121 accessions was carried out in the spring of 2007 at Zhengzhou, China (34°16’ N, 112°42’ E) in the open field. Germinated seeds were vernalized at 4 °C in the dark for 25 d before they were sown in pots in a growth chamber at 25/20 °C (day/night) with a 16 h photoperiod, or in the open field. Five individuals for each accession were planted in the growth-chamber trial and 15 in the open-field trial. For the open-field trial, vernalized germinated seeds were sown in pots that were placed in a greenhouse on 14 March 2007, and then transplanted into the open field in a randomized design across three blocks on 21 March 2007.

For the DH population, five germinated seeds from each line were treated at 6 °C in the dark for 25 d and then sown in the growth chamber under a 16 h photoperiod at 25/20 °C (day/night). In the open field trial, 15 non-vernalized germinated seeds from each line were sown in pots under an unheated plastic tunnel on 19 January 2005 and the plants were transplanted to the open field in Zhengzhou on 21 March 2005. Mean temperatures of 5–8 °C for the period from 20 January to 20 February and 8–12 °C for the period from 21 February to 20 March were recorded.

### Flowering-time evaluation

Flowering time was measured as days to flowering (DTF) similar to the method described by Werner et al. (2005). For the collection of 121 *B. rapa* accessions, DTF was recorded for up to 60 d. Plants without flower buds at the end of the experiment were assigned a value of 60 DTF. For the DH population, DTF of each line was investigated since the experiment lasted until plants of the started line flowered.

### BrFLC1 amplification and CAPS marker analysis

Specific primers were designed for *BrFLC1* (http://ncbi.nlm.nih.gov; AY115678). The forward primer was FLC1F4 (5'-CTTGAGGGAATCAAATGTGCATAA-3') and the reverse primer was FLC1R1 (5'-CCATATCTAGCTTCGGCTCG-3'). The amplified fragment covers

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**Table 1.** Overview of *B. rapa* accessions used in this study sorted according to cultivar group

| Cultivar group       | No. of DH lines | No. of inbred lines | No. of hybrids | Total |
|----------------------|-----------------|---------------------|----------------|-------|
| Chinese cabbage      | 71              | 3                   | 0              | 74    |
| Pak choi             | 19              | 0                   | 0              | 19    |
| Yellow sarson        | 5               | 1                   | 0              | 6     |
| Caxin                | 4               | 0                   | 1              | 5     |
| Turnip               | 0               | 3                   | 1              | 4     |
| Wutacai              | 0               | 1                   | 2              | 3     |
| Zicaitai             | 1               | 0                   | 2              | 3     |
| Taicai               | 0               | 3                   | 0              | 3     |
| Mizuna               | 0               | 2                   | 0              | 2     |
| Rapid cycling        | 0               | 1                   | 0              | 1     |
| Broccolletto         | 0               | 1                   | 0              | 1     |
| Total                | 100             | 15                  | 6              | 121   |

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**Table 2.** List of 30 sequenced *B. rapa* accessions used in this study

| Accessions  | Name          | DTF | Cultivar group  | Type   |
|-------------|---------------|-----|-----------------|--------|
|             |               | Growth chamber | Open field    |        |
| FL01        | Y411-3        | 60  | 51±1.1          | Chinese cabbage |
| FL13        | Y152-9        | 33±0.6 | 37±1.1          | Chinese cabbage |
| FL07        | R16-11        | 32±0.0 | 31±2.2          | Chinese cabbage |
| FL15        | Y177–12       | 38±0.4 | 38±0.0          | Chinese cabbage |
| FL16        | Y195–93       | 33±0.9 | 34±0.9          | Chinese cabbage |
| FL27        | Y392–16       | 38±2.2 | 38±0.9          | Chinese cabbage |
| FL45        | Y538–3        | 40±2.2 | 40±2.8          | Chinese cabbage |
| FL56        | BDC5186-7     | 33±0.8 | 26±2.2          | Pak choi |
| FL77        | N26-2         | 60   | 60              | Pak choi |
| FL66        | N1-1          | 60   | 60              | Pak choi |
| FL73        | N14-17        | 60   | 60              | Pak choi |
| FL78        | N29-168       | 33±0.4 | 32±0.0          | Pak choi |
| FL59        | YN-1          | 24±5.2 | 19±0.0          | Pak choi |
| FL111       | P143          | 28±0.0 | 22±0.0          | Yellow sarson |
| FL86        | DH38-65       | 32±0.0 | 32±0.9          | Yellow sarson |
| FL89        | DH30-65       | 32±0.0 | 30±0.4          | Yellow sarson |
| FL91        | DH3-67        | 37±0.0 | 36±0.0          | Yellow sarson |
| FL95        | L58-1         | 25±4.0 | 22±0.0          | Caxin  |
| FL65        | F041397-3     | 30±1.9 | 27±0.0          | Zicaitai |
| FL101       | Glu002        | 36±0.1 | 35±0.1          | Wutacai |
| FL96        | L144          | 18±0.0 | 19±0.0          | Rapid cycling |
| FL102       | Glu004        | 37±0.0 | 34±1.1          | Komatsuna |
| FL104       | Glu009        | 32±0.0 | 32±0.0          | Broccolletto |
| FL107       | Glu107        | 27±4.0 | 19±0.0          | Spring turnip |
| FL106       | Glu087        | 60   | 60              | Fodder turnip |
| FL110       | P115          | 32±0.0 | 32±0.0          | Vegetable turnip |
| FL148       | Glu018        | 60   | 59±4.9          | Winter turnip |
| FL103       | Glu007        | 41±2.8 | 38±1.0          | Mizuna  |
| FL142       | B06038-4      | 60   | 60              | Taicai  |
| FL109       | Z052954       | 45±4.0 | 55±6.9          | Taicai  |
the region of exons 4–7 and the intervening introns between these exons. Genomic DNA was isolated from lyophilized young leaves as described by Wang et al. (2005). PCR was carried out in a total volume of 20 μl containing 50 ng template DNA, 0.5 μM of each primer, 200 μM of dNTPs, 1× PCR reaction buffer, and 1 U Taq polymerase. PCR was performed under the following conditions: the template was denatured at 94 °C for 3 min, followed by 35 cycles of amplification (94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min 30 s), and a final extension at 72 °C for 7 min. PCR products from the 30 accessions listed in Table 2 were purified by QIAquick gel extraction kits (Qiagen) and sequenced directly. For CAPS analyses, the amplicons from the 30 accessions listed in Table 2 were sequenced for each fragment.

*RNA extraction and reverse-transcriptase PCR (RT-PCR)*

Germinated seeds from three Chinese cabbage and three pak choi accessions were vernalized at 4 °C for 25 d and then planted in pots in the growth chamber at 25/20 °C (day/night) with a 16 h photoperiod. Young leaves were collected from plants after they had been in the growth chamber for 42 d. Total RNA of leaves was extracted using the RNeasy Plant Mini Kit (QIAGEN, http://www.qiagen.com). First-strand cDNA was synthesized from 1 μl total RNA by using a cDNA synthesis kit (MBI fermentas, http://www.fermentas.com) according to the manufacturer’s instruction. RT-PCR of *BrFLC1* was performed as described above for genomic PCR using gene-specific primers BrKFLC1F and FLC1R1. The forward primer BrKFLC1F (5'—CGCAAGACTGTTGGAGA-3') was designed from the 5' UTR (Kim et al., 2007) and the reverse primer FLC1R1 (5’—CCATATTATCAGCTTCGGCTCG-3') was in exon 7. The amplified products were separated on ethidium bromide-stained 2.0% agarose gels, purified by QIAquick gel extraction kits (Qiagen), and then cloned into the pGEM-Teasy vectors (Promega, http://www.promega.com) for sequencing. Two independent clones were sequenced for each fragment.

*Sequence analysis*

Sequencing of the *BrFLC1* gene was performed on an ABI 3730XL DNA analyser (Perkin-Elmer, USA). All sequences were aligned against the published *BrFLC1* sequence. Sequence alignment and analysis were conducted using multiple sequence alignment of DNAman ver.5.2.2 (Lynnon, http://www.lynnon.com).

*Statistical analysis*

Analysis of variance (ANOVA) and analysis of association were tested by one-way ANOVA and one-tailed Pearson correlation in SPSS version 12.0.1 statistical package (SPSS Inc., Chicago, IL, USA). ANOVA was performed with marker genotype as factor.

**Results**

*Phenotypic variation in flowering time*

A wide range in flowering times was observed in the vernalized seedlings of 121 accessions grown both in the open field and in the growth chamber. The flowering time varied from 19 to 60 DTF and from 18 to 60 DTF in the open field and growth chamber trials, respectively (Fig. 1). These two trials were significantly correlated with *r*=0.913; *P* <0.001. At 60 DTF, six accessions in the open field trial and 26 accessions in the growth chamber did not flower.

Thirty accessions with a wide flowering-time variation and representing different cultivar groups were selected for sequence analysis of *BrFLC1* (Table 2). Five accessions in the open field and seven accessions in the growth chamber had not yet flowered at 60 DTF. The average DTF was 37.9±2.5 in the open-field trial and 39.2±2.3 d in the growth-chamber trials. Data from these two trials were significantly correlated (*r*=0.966; *P* <0.001).

*Nucleotide polymorphisms at the BrFLC1 gene*

To pursue the allelic variation in *BrFLC1* further, a 980 bp fragment was amplified from *B. rapa* genomic DNA with primer combination of FLC1F4 in exon 4 and FLC1R1 in exon 7. The amplified fragments from the 30 selected accessions were sequenced with the reverse primer FLC1R1. In total, nucleotide polymorphisms were identified at seven sites within the amplified region after multiple sequence alignment, and all these polymorphisms were SNPs (single-nucleotide polymorphisms). For each polymorphism, a name was designated with an initial ‘P’ (position) followed by ‘i’ (intron) combined with the serial number of the intron position of the variable nucleotide in the intron. Two nucleotide polymorphisms located at intron 5 were designated as Pi5+104 and Pi5+201, while the other five polymorphisms in intron 6 were Pi6+416, Pi6+349, Pi6+392, Pi6+416, and Pi6+428 (Fig. 2). No exon polymorphisms were observed in the amplified region.

**Fig. 1.** Frequency distribution of flowering-time phenotype in the collection of 121 *B. rapa* accessions. The plants were grown in the open field (OF) or in the growth chamber (GC) after 25 d of vernalization of germinated seeds. 60NF indicates that the plants did not show flower buds after 60 d.
Association between flowering time and nucleotide polymorphisms in **BrFLC1**

To dissect the role of **BrFLC1** allelic variation, the association between alleles resulting from the seven nucleotide polymorphisms and flowering-time phenotype was analysed across the 30 sequenced accessions. The results indicated that two polymorphisms, Pi5+104 and Pi6+1, were significantly correlated with flowering-time phenotype. The correlation was consistent in both the growth chamber (Pi5+104 polymorphism, \( r = 0.756, P < 0.001 \); Pi6+1 polymorphism, \( r = 0.744, P < 0.001 \), and the open field (Pi5+104 polymorphism, \( r = 0.713, P < 0.001 \); Pi6+1 polymorphism, \( r = 0.695, P < 0.001 \)). When tested by ANOVA, the mean DTF for accessions with Pi6+1(G) allele is significantly later than those with the Pi6+1(A) allele (\( P < 0.001 \)), with a delay of 20.5 d in the open-field and 17.4 d in the growth-chamber trial (Table 3). For the Pi5+104 polymorphism, the mean DTF for accessions with the C allele is significantly later than those with the T allele (\( P < 0.001 \)), with a delay of 20.1 d in the open-field and 18.0 d in the growth-chamber trial. The significant correlation between nucleotide polymorphisms and flowering-time phenotype indicated that variations at Pi5+104 and Pi6+1 could affect flowering time in *B. rapa* accessions.

As the Pi6+1 polymorphism is located at +1 bp in intron 6 and had either a G (Pi6+1(G) allele) or an A (Pi6+1(A) allele) nucleotide substitution, it was inferred that the base substitution of G\( \rightarrow \)A at this site may alter the splicing site specificity, resulting in alternative splicing and producing at least two different transcripts. The Pi6+1 polymorphism affects RNA splicing

To test whether the base substitution at Pi6+1 altered RNA splicing, RT-PCR analysis of **BrFLC1** transcripts from six accessions, four with the Pi6+1(G) allele and two with the Pi6+1(A) allele was performed (Table 4). The forward primer was BrKFLC1F located in the 5' UTR and the reverse primer was FLC1R1 located in exon 7. All accessions with the Pi6+1(G) genotype gave rise to a single band, while the Pi6+1(A) genotype accessions produced two bands (Fig. 3). This indicated that the base substitution of G\( \rightarrow \)A at Pi6+1 in the **BrFLC1** gene changed splicing specificity, resulting in alternative splicing and producing at least two different transcripts.

The RT-PCR **BrFLC1** fragments from the six accessions were sequenced, and four splicing patterns, including one constitutively spliced (SpG, G genotype at the Pi6+1 site) and three alternatively spliced transcripts (SpA1–SpA3) were identified (Fig. 4). Among the three alternative splicing patterns, two (SpA1 and SpA2) resulted in longer transcripts because the first 25 or 55 nucleotides of exon 6 were retained in the final transcript. This led to a frame shift and a predicted stop codon at 12–14 nt in exon 7 for both SpA1 and SpA2. As a result, alternatively truncated proteins were likely to be produced. By contrast, SpA3 results in a shorter transcript due to an alternative splice acceptor site, leading to a deletion of the entire exon 6.
In addition, the constitutive (wild-type) splicing pattern SpG was only detected in the accessions with the Pi6+1(G) allele, while in the accessions with the Pi6+1(A) allele, all three alternative splicing patterns, SpA1–SpA3, were detected (Table 4). Interestingly, in the accessions with the Pi6+1(A) allele, SpA3 was always detected in combination with one of the other two alternative splicing patterns, SpA1 or SpA2 (Table 4).

Confirmation of the relationship between splice-site mutation of BrFLC1 and variation in flowering time in the collection of 121 B. rapa accessions

As the Pi6+1 genotype is associated with flowering-time variation in B. rapa, it would facilitate the identification of the flowering-time type if a molecular marker could be developed to reveal the polymorphism at this site. In the constitutive allele, Pi6+1(G) is in the recognition site for restriction enzyme MvaI. This character was used to develop a CAPS marker to detect the Pi6+1 genotype. Although another MvaI restriction site was located at 41 bp in exon 4 (Fig. 5A), the small fragment produced by cutting this site could not be observed on 1% agarose gel. The 30 sequenced accessions were first analysed for this CAPS marker and showed two banding patterns. A band of about 940 bp was observed for accessions with the Pi6+1(A) genotype, while the accessions with the Pi6+1(G) genotype showed a band at about 500 bp (Fig. 5B). Sequence analysis revealed that the band observed in the Pi6+1(G) genotype actually included two fragments of 449 bp and 491 bp. This CAPS marker was designated as FLC1-MvaI.

The remaining 91 accessions with recorded flowering-time phenotypes were genotyped with CAPS FLC1-MvaI to establish the correlation between the splicing site genotype and flowering-time phenotype. In total, 68 accessions had the Pi6+1(A) allele and 53 accessions had the Pi6+1(G) allele. There was a tendency for a relatively late flowering time in Pi6+1(A) accessions and a relatively early flowering time in Pi6+1(G) accessions, in both the open field (Fig. 6A) and the growth chamber (Fig. 6B). The association between the CAPS marker genotype and flowering-time phenotype across 121 B. rapa accessions was calculated. The results revealed that marker genotype was significantly correlated with flowering-time phenotype both in the open-field ($r$=0.681; $P$ <0.001) and growth-chamber trials ($r$=0.654; $P$ <0.001), respectively. When tested by ANOVA, mean DTF for accessions with the Pi6+1(G) allele is significantly later than those with the Pi6+1(A) allele (($P$ <0.001), with a delay of 13.8 d in the open-field trial and 15.4 d in the growth-chamber trial (Table 3). To exclude the possibility that the flowering-time difference is due to the genetic background of the materials, the flowering-time phenotype was also tested between different cultivar groups by ANOVA.

To test the influence of the population structure on flowering-time variation, the materials were grouped into three groups, Chinese cabbage (CC, 74 accessions), pak choi (PC, 29 accessions), and others (O, 18 accessions) (Zhao et al., 2007). The splice-site mutation occurred in all of these groups, in 35 accessions of the CC group, in 22 accessions of the PC group, and in 11 accessions of the third group. The result showed that there was no significant difference of flowering time among the three groups. However, within each group, flowering time was significantly different between the two genotypes and was significantly correlated with the genotypes ($r$=0.521–0.859; $P$ <0.001). These results indicated that the correlation between the splicing site variation and flowering time is not due to population structure.

The association between early-flowering accessions with the A allele and late-flowering accessions with the G allele at the Pi6+1 site was not absolute, and several exceptions were identified (Fig. 6). Flowering time of accessions with the G allele had a mean DTF of 50.2 (lower limit 47.1; $P$ <0.01) in the growth-chamber trial, and 45.9 (lower limit...
The effect of the splicing mutation at Pi6+1 of BrFLC1 on flowering time was influenced by vernalization

The involvement of the splicing variation of BrFLC1 in flowering time under vernalized conditions was further confirmed using the Chinese cabbage DH population BrIVFhn. One parental line Y177 is late flowering and has the G allele, while another parental line Y195 is relatively early flowering and has the A allele. The DH population was genotyped with the CAPS marker FLC1-\textit{Mva} and was phenotyped in the open field and in the growth chamber. In the growth-chamber trial, a significant difference ($P<0.001$) in flowering time was detected between the lines with the G and A alleles at Pi6+1, indicating a FT_QTL at this locus. When analysed with MAPQTL 4.0, it showed that the QTL localized on A10 (LOD=4.71) at the position of the BrFLC1 gene (Schanze et al., 2002), with explained phenotypic variance of 7.5% (X-W Wang et al., unpublished data). However, in the open-field trial no significant difference of flowering time was detected between lines with the two different alleles at Pi6+1 (Table 3). Vernalization in the field trial was longer than in the growth-chamber trial. The difference in detecting the effect of the BrFLC1 alleles on flowering time under these two conditions suggested that BrFLC1 is a putative candidate gene for an FT_QTL influenced by vernalization status.

**Discussion**

Variation in a BrFLC1 splicing site was discovered in a panel of natural B. rapa accessions with a wide range of variation in flowering time, and its relationship with flowering-time phenotype was investigated both in a collection of 121 accessions and a DH population. The transition of G→A at the first nucleotide site in intron 6 altered splicing and gave rise to aberrant splicing products, which was associated with early flowering in B. rapa. A CAPS marker was developed to facilitate the detection of the splice-site variation and its use has been validated for flowering-time prediction in a collection of 121 B. rapa accessions and a Chinese cabbage DH population with 180 individuals.

**Involvement of BrFLC1 in the control of flowering time**

In B. rapa, previous studies on QTL analyses of flowering time suggested BrFLCs as candidate genes underlying flowering-time QTL (Osborn et al., 1997; Kole et al., 2001; Schranz et al., 2002; Lou et al., 2007), and four FLC homologues, BrFLC1, BrFLC2, BrFLC3, and BrFLC5, have been cloned in B. rapa, and mapped on linkage groups A10, A02, A03, and A03, respectively (Kim et al., 2006). In B. oleracea, this is less evident, only BoFLC2 probably contributes to the control of flowering time (Okazaki et al., 2007). In B. nigra, CO and COL, and not FLC, were suggested as candidate genes underlying flowering-time QTL (Axeisson et al., 2001; Lagercrantz et al., 2002; Osterberg et al., 2002). In the present study, evidence was obtained using both a collection of B. rapa accessions and a DH population, suggested that the splicing-site mutation in BrFLC1 is significantly associated with flowering-time variation.

**Discovery of different splicing patterns of BrFLC1**

To date, six different alternative FLC transcripts have been discovered in Arabidopsis. Werner et al. (2005) found two FLC variants that were alternatively spliced. The Van-0 accession displayed exon 6 skipping, while the Bur-0 accession displayed a partly retained exon 6 resulting in a premature stop codon in exon 7. Three natural FLC alleles with severely affected protein function in accessions Cen-0 (part of exon 7 skipped), Li-2 (exons 2–7 skipped), and Cal-0 (partly retained intron 6 resulting in premature stop codon in intron 6 and deletion of exon 7) were discovered (Lempe et al., 2005). In the Est-0 and Le-0 accessions, an alternatively spliced FLC transcript that uses a nonconsensus cryptic splice donor site in intron 1 (GA)

![Fig. 6. Distribution of flowering time and CAPS marker genotype in a collection of B. rapa germplasm (n=121). (A) Plants were grown in the open field; (B) plants were grown in the growth chamber. Black and white columns represent accessions with G and A alleles, respectively, at the Pi6+1 site of BrFLC1. Arrows indicate mean values of lines with an A or G allele. NF indicates lines that did not flower during the experiment.](image-url)
and splice acceptor site in intron 6 (CT) was observed only after vernalization, which contains all of the first and last exon of the FLC gene, as well as 73 bp of intron 1 and 15 bp of intron 6 (Caicedo et al., 2004). In B. rapa, two alternate splice variants of BrFLC5 were observed by Schranz et al. (2002). However, the relationship between these variations and flowering was not clarified. In the present study, besides the constitutive SpG splicing pattern, three alternative variants of BrFLC1 mRNA splicing products, named SpA1, SpA2, and SpA3 were identified (Fig. 3). For SpA1 and SpA2, different parts of intron 6 are retained, similar to the alleles in Arabidopsis Bur-0 (Werner et al., 2005); whereas for SpA3, exon 6 is skipped, similar to the alleles in Arabidopsis Van-0 (Werner et al., 2005). However, the coexistence of two different alternatively splicing patterns in one accession was not only different from previous reports of single alternative splicing pattern for fad3c (exon skipping) in B. napus (Hu et al., 2007), BoGSL-ELONG (intron retention) in B. oleracea (Li and Quiros, 2002), and FLC (exon skipping) in Arabidopsis (Lempe et al., 2005), but also different from the co-existence of constitutive splicing and alternative splicing patterns observed for FLC in Arabidopsis (Caicedo et al., 2004).

Accessions having flowering-time phenotype conflicting with the Pi6+1 allele

The significant association of G→A splicing site genotype with flowering-time phenotype across 121 B. rapa accessions found in the present study revealed that the splicing site mutation in BrFLC1 correlated with early flowering in B. rapa. There was a general association of early-flowering accessions with the A allele and late-flowering accessions with the G allele at the Pi6+1 site, but this relationship was not absolute, since several exceptions to this rule in the growth-chamber and open-field trials were identified. Different explanations are feasible. First, variation located at other regions of BrFLC1 besides the studied region of exons 4–7 can also affect flowering. It has been reported that the large intron 1 of FLC contains many sequence polymorphisms and has been implicated in the control of FLC expression (Caicedo et al., 2004). Secondly, multiple functional FLC loci are involved in the variation of flowering time in Brassica species. Each of the five FLC copies from B. napus delayed the flowering time significantly in Arabidopsis (Tadege et al., 2001) and three (BrFLC1, BrFLC2, and BrFLC5) of four FLC homologues from B. rapa co-segregated with flowering-time loci (Schranz et al., 2002). BrFLC2 on A02 and BrFLC1 on A10 co-localizing with flowering-time-QTL were also identified in multiple populations of B. rapa (Lou et al., 2007). BrFLC2 and BrFLC3 are functional in flowering-time control besides BrFLC1 (Kim et al., 2007). Therefore, late flowering in accessions with the Pi6+1(A) allele can be explained by the compensation of the non-functional BrFLC1 allele by functional allele(s) of other FLC homologues. In addition to FLC, many other flowering-time genes involved in the vernalization pathway in Arabidopsis affect flowering. Although FLC is responsible for a considerable part of the vernalization response in Arabidopsis, nonetheless, two other FLC-independent vernalization responses, the AGL24- and AGL19-branches, have been identified (Alexandre and Hennig, 2008). Similar to FLC, its homologous MAF genes are usually regulated by vernalization (Alexandre and Hennig, 2008). Finally, besides genes involved in the vernalization pathway, other vernalization-independent flowering-related genes or genes involved in other flowering-promotion pathways may alter the flowering-time phenotype, similar to what has been found for the CO gene in B. nigra (Osterberg et al., 2002), which is under complex genetic control (Simpson et al., 1999; Boss et al., 2004).

Influence of vernalization on the effect of BrFLC1

The correlation of Pi6+1 genotypes and the flowering-time phenotype was confirmed by testing a DH population grown after partial vernalization (germinated seeds kept at under 6 °C for 25 d), but it could not be confirmed when the population was grown under a sustaining natural vernalization condition. Previous research showed that cold treatment of Chinese cabbage plants at 4 °C for 61 d is sufficient to induce flowering of most Chinese cabbages (Ajisaka et al., 2001), and vernalization at 4–6 °C for 2 weeks induced flowering time in many Chinese cabbage accessions (Zhao et al., 2007). Gene expression of BrFLC1 and BrFLC2 was still detected in late-flowering plants even after 40 d of vernalization (Kim et al., 2007), and expression of BrFLC in high non-bolting Chinese cabbage cultivars remained detectable after 7 weeks of vernalization treatment but was almost undetectable after 9 weeks of vernalization treatment (Li et al., 2005). In the present study, plants grown in a growth chamber following cold treatment at 6 °C for 25 d were regarded as partially vernalized. In this case, expression of BrFLC1 is not completely suppressed, and the effect of the functional Pi6+1(G) allele on flowering-time delay is detected. By contrast, when the plants were grown in the open field in Zhengzhou in early spring, the long period of low temperature (mean temperatures of 5–8 °C for the period from 20 January to 20 February and 8–12 °C for the period from 21 February to 20 March) during the growing season may completely block the expression of BrFLC1 and thus the different alleles are not expressed and the repressive effect of Pi6+1(G) is undetectable.

Application of FLC1-Mva to breeding programmes for late-flowering B. rapa by marker-assisted selection

In Asian countries where Chinese cabbage and pak choi are widely cultivated, B. rapa crops are subjected to risk of exposure to low temperature in early spring which may lead to loss of yield because of premature flowering. Breeding of cultivars with late flowering is an important step for achieving a good yield of B. rapa crops during this season. A co-dominant marker FLC1-Mva I applicable to marker-assisted breeding programs was successfully developed for identification of early-flowering genotypes with the Pi6+1(A)
allele and late-flowering genotype with the Pi6+1(G) allele of BrFLC1. Marker-assisted selection of seedlings will be very valuable, because phenotypic selection requires a very long time from sowing to flowering.

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