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A transposon insertion in *FLOWERING LOCUS T* is associated with delayed flowering in *Brassica rapa*

Xueming Zhang[a,1], Lin Meng[a,1], Bo Liu[a], Yunyan Hu[a], Feng Cheng[a], Jianli Liang[a], Mark G.M. Aarts[b], Xiaowu Wang[a], Jian Wu[a,*]

[a] Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Zhongguancun Southern Street 12, 100081 Beijing, China
[b] Laboratory of Genetics, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, the Netherlands

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ABSTRACT

Long days and vernalization accelerate the transition from vegetative growth to reproductive growth in *Brassica rapa*. Bolting before plants reach the harvesting stage is a serious problem in *B. rapa* vegetable crop cultivation. The genetic dissection of flowering time is important for breeding of premature bolting-resistant *B. rapa* crops. Using a recombinant inbred line (RIL) population, we twice detected two major quantitative trait loci (QTLs) for flowering time in two different growing seasons that were located on chromosomes A02 and A07, respectively. We hypothesized that an orthologue of the *Arabidopsis thaliana FLOWERING LOCUS T* (*FT*) gene, named as *BrFT2*, was the candidate gene underlying the QTL localized to A07. A transposon insertion in the second intron of *BrFT2* was detected in one of the parental lines, which was predicted to generate a loss-of-function allele. Transcription analysis revealed that the *BrFT2* transcript was not present in the parental line that harbored the mutated allele. RILs carrying only the mutated *BrFT2* allele showed delayed flowering regardless of growing seasons when compared to RILs carrying the wild-type *BrFT2* allele. These data suggest that *BrFT2* is involved in flowering time regulation in controlling flowering time in *B. rapa*.

1. Introduction

The switch from vegetative to reproductive growth marks a major developmental transition in flowering plants. Controlling the timing of this transition is especially important in crop plants, as correct timing ensures flowering under optimal conditions that maximize reproductive success and seed production. Furthermore, the absence or delay in flowering is especially important to leafy vegetables. Floral transition is highly responsive to environmental cues. The regulation of flowering time, including its associated network, has been extensively studied in the model plant species *Arabidopsis thaliana* [1–5]. Over 180 *A. thaliana* genes are implicated in flowering time control based on characterization of loss-of-function mutants or analysis of transgenic plants [3]. Six major pathways control flowering-time in *A. thaliana*: the photoperiod/circadian clock pathway, vernalization pathway, ambient temperature pathway, age pathway, autonomous pathway, and gibberellin pathway [3,6]. The photoperiod response to changes in day length and the vernalization response to low temperatures are two major pathways that regulate flowering time in *A. thaliana* [5]. In long-day (LD) conditions, a plant produces a series of signals in the leaves in response to light, which involve the *CONSTANS* (*CO*), *GIGANTEA* (*GI*), and *FLAVIN KELCH F BOX 1* (*FKF1*) genes. *GI* and *FKF1* are upstream genes of *CO*, and their interaction releases repression of *CO* mRNA transcription by inducing degradation of the transcriptional repressor *CYCLING DOF FACTOR1* (*CDF1*) [7]. CO plays a key role in the photoperiod pathway by activating the transcription of the floral integrator gene *FLOWERING LOCUS T* (*FT*). *FT* is expressed within the distal part of the leaf, and its encoded protein moves through the phloem to the meristem, thus acting as a long-distance systemic signal between leaves and the shoot meristem [8–10]. Without exposure to a low winter temperature, plants express the *FLOWERING LOCUS C* (FLC) protein, which directly binds to the promoter of the *FT* gene in the shoot apical meristem (SAM) and vascular tissues, to repress expression and delay flowering [11,12]. In the SAM, FT interacts with the bZIP transcription factor FD to form a FT/FD heterodimer complex [13,14]. This activates

* Corresponding author. Fax: +86 10 62174123.
E-mail addresses: yimingzhangbp@163.com (X. Zhang), mlbio@126.com (L. Meng), lb_bobo@aliyun.com (B. Liu), huyunyanspring@126.com (Y. Hu), chengfeng@caas.cn (F. Cheng), liangjianli@caas.cn (J. Liang), mark.aarts@wur.nl (M.G.M. Aarts), wangxiaowu@caas.cn (X. Wang), wujian@caas.cn (J. Wu).
[1] These authors contributed equally to this manuscript.

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expression of the floral meristem identity genes *APETALA 1* (*AP1*) and *FRUITFUL* (*FUL*), which together initiate the development of flower buds [9,13–16].

Brassica is a large and diverse genus that includes various important oil, vegetable, fodder, and condiment crops; it is closely related to *A. thaliana*. The genus underwent a genome triplication event that preceded the origin of the diploid species *Brassica rapa* (AA), *Brassica oleracea* (CC), and *Brassica nigra* (BB) [17]. Cultivated varieties of *B. rapa* exhibit extreme developmental and morphological diversity and are generally divided into leafy, turnip, and oil types based on their morphological appearance and the organs consumed [18,19]. *B. rapa* crops are normally grown in autumn and spring seasons. In *B. rapa* leafy vegetables such as Chinese cabbage and pak choi, bolting can occur before plants reach the harvest stage; this mostly occurs in the spring season because of low temperatures at the beginning of cultivation and longer day lengths during the growing period. Genetic dissection of flowering time control is important for breeding late-bolting leafy *B. rapa* cultivars. Mesopolyploidy has imparted contrasting effects on genes involved in flowering time control; multiple copies of *FLC* and *VERNALIZATION1* (*VRN1*) produced by whole genome triplication have been preserved in the *B. rapa* genome, whereas the GI and the three *CONSTANS-LIKE* (*COL*) genes are limited to only one copy [17]. In *B. rapa*, the most studied flowering time control gene is FLC. There are four *FLC* paralogues in *B. rapa* (*BrFLC1*, *BrFLC2*, *BrFLC3*, and *BrFLC5* [20]), three of these have been confirmed to be involved in flowering time control [21]. Naturally occurring mutations responsible for flowering time variation have been identified in the *BrFLC1* and *BrFLC2* [22–24]. Quantitative trait locus (QTL) and expression quantitative trait loci (eQTLs) analyses have revealed that the *BrFLC2* locus plays a strong role in flowering time regulation in *B. rapa* [25,26], whereas *BrFLC1* has been identified as a potential candidate gene affecting transgenerational regulation of flowering time and seed germination [27]. Lou et al. [28] explored the correlation between circadian rhythms and flowering time in *B. rapa* by assessing co-localization of the QTLs for the two traits. Although a list of candidate genes was proposed for these QTLs based on microsynteny between *B. rapa* and *A. thaliana*, no direct effect of sequence variation on the function of those genes was detected. To date, no *B. rapa* genes involved in any of the other flowering time regulation pathways, as well as the contribution of other regulation pathways to flowering time variation have been identified.

In the present study, QTL analyses for flowering time in *B. rapa* were performed using a recombinant inbred line (RIL) population. Candidate genes for identified QTLs were proposed and confirmed using gene-specific markers, re-sequencing, and gene expression profiling.

2. Materials and methods

2.1. Plant material and flowering time evaluation

A *B. rapa* RIL population (F_7) produced from a cross between a caixin line (L58, *ssp. parachinensis*) and a yellow sarson line (R-o-18, ssp. *tricolaris*) [29] was used for QTL mapping of flowering time variation. A total of 149 F_7 lines were used for map construction and phenotyping. Seeds of the parental lines and the RILs were germinated and sown in pots filled with potting soil and fertilizer, and grown in a greenhouse without climate control from May 1, 2011 to July 1, 2011, and in the same greenhouse from September 1, 2012 to December 4, 2012, in the Haidian District of Beijing, China. The monthly day length was 447 and 449 h in May and June, respectively, whereas in Beijing, it was 373, 345, 299, and 290 h in September, October, November, and December, respectively. The temperatures in the greenhouse also varied between the two growing seasons. During the spring experiment, the daily mean temperatures varied from 20 °C to 32.5 °C in the greenhouse, whereas in the autumn growing season, variation in temperature ranged from 13.5 °C to 28.5 °C. Six replicates were planted for each line in a randomized complete design. Ten lines were not phenotyped in the experiment in 2011, and 13 lines in 2012 due to limited F_7 seeds. Flowering-time was defined as days from sowing to appearance of the first bud (days to flowering, DTF). Plants that did not bud by the end of the experiment were assigned a value of 62 DTF when sown in spring 2011, and 95 DTF in autumn of 2012. For RNA isolation, seeds of the parental lines were sown in the same greenhouse on June 23, 2015. Leaves from one-month-old plants were harvested for RNA isolation.

2.2. Re-sequencing, and InDel marker development and analysis

Genomic DNA was extracted from leaf samples using the CTAB method [30]. Paired-end (PE) re-sequencing data were generated using an Illumina HiSeq™ 2000 platform (Illumina Inc., San Diego, CA, USA), producing reads encompassing 3 Gb (~6× genome coverage) of the L58 genome and 2.8 Gb (~5.6× genome coverage) of that of R-o-18. The *B. rapa* genome sequence was retrieved from the *Brassica* database (BRAD; http://brassicadb.org) and used as reference. PCR-based insertion/deletion (InDel) markers were developed using the pipeline developed by Liu et al. [31]. Genotyping of the InDel markers was performed using the procedure described by Wang et al. [32]. In addition, a gene-specific InDel marker of *BrFLC2* was screened for polymorphisms between the parents and among the RILs, as described by Wu et al. [23].

2.3. Genetic mapping

A total of 386 polymorphic InDel markers were genotyped in 147 RILs. Linkage analysis and genetic map construction were performed using the JoinMap 4.0 software with a minimum logarithm of odds (LOD) score of ≥5.0 (Van Ooijen [34]). The Kosambi map function was used to estimate the genetic distance between markers [33].

MAPQTL 4.0 software (http://www.kyazma.nl) was used for QTL analysis. Putative QTL was identified using interval mapping (IM) and multiple-QTL model (MQM) mapping methods. Initial IM analysis was performed to find putative QTLs [34]. Then, MQM analysis was performed to precisely locate QTLs after automatic selection of cofactors within the vicinity of the QTL. A permutation test was applied to each data set (1000 repetitions [35]) to obtain genome-wide LOD thresholds ($P = 0.05$), which revealed that a LOD value of 3.00 was a significant threshold in both experiments. A mapping-step size of 1 cM was used for both IM and MQM analyses. QTL positions were estimated as the position with a maximum LOD score on a linkage group. Two-LOD support intervals were established as 95% confidence intervals [36].

2.4. Amplification and sequencing of BrFTs

Specific primers were designed to amplify two syntenic orthologues of *FT* in *B. rapa*, *BrFT1* (Bra022475) and *BrFT2* (Bra004117), based on the *B. rapa* genome sequence (Table 1, Fig. 1). PCR amplification was conducted in a total volume of 20 μL, containing 50 ng of template DNA, 0.5 μM of each primer, 200 μM dNTPs, 1× PCR reaction buffer, and 1 U of *Taq* polymerase (TransGen Biotech, http://www.transgen.com). PCR was conducted on a thermocycler (Applied Biosystems®, Waltham, MA, USA) using the following conditions: denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min 30 s; and a final extension at 72 °C for 10 min. Amplified products were separated on SYBR green-stained 1.5% agarose gels. Purified PCR

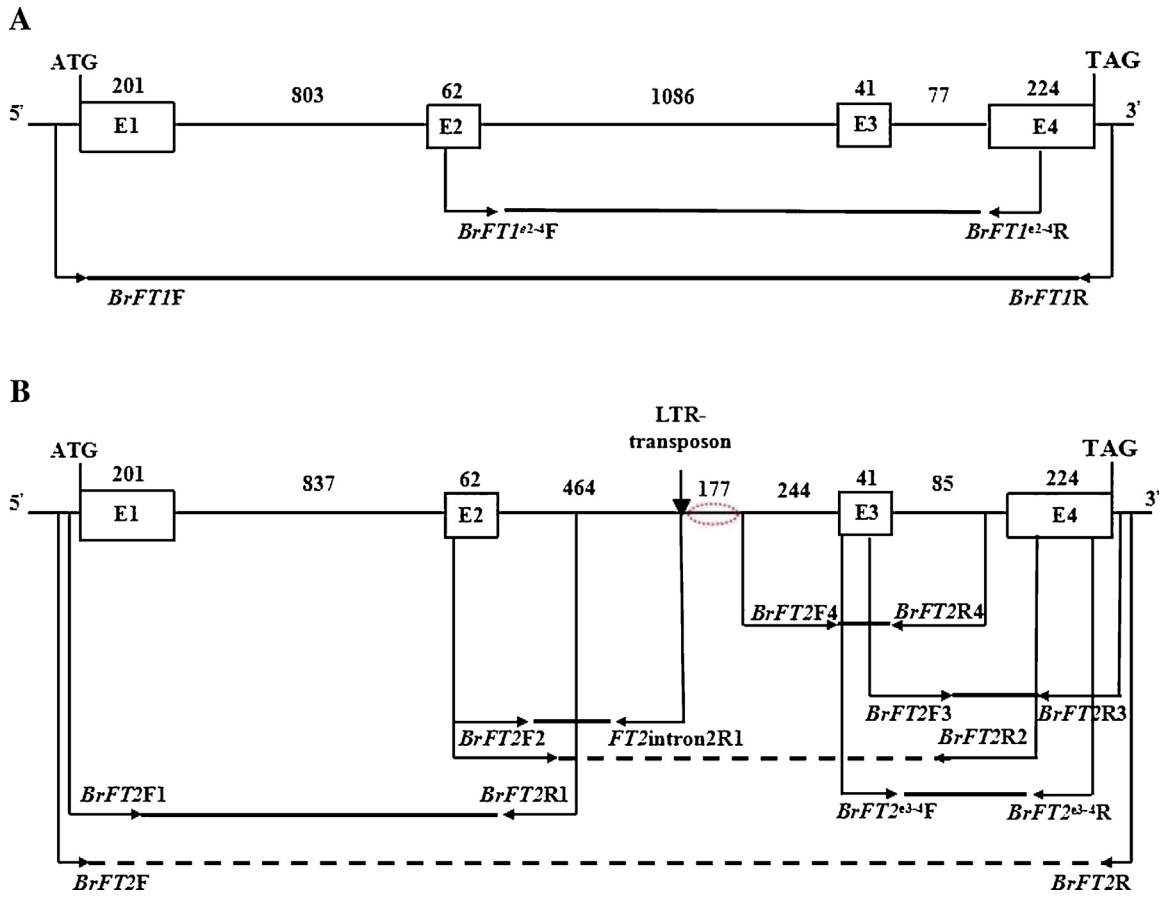

Fig. 1. The primers used in this study.
E, exon; the exon sizes are indicated in the white boxes, the number above the black line indicates intron size. (A) *BrFT1*, (B) *BrFT2*. The solid line indicates the primer pair used in the amplification of the fragment. The dashed line indicates the primer pair that could not amplify the fragment in R-o-18. The arrow indicates the site where the LTR-transposon inserted. The dashed circle indicates the fragment that was not amplified in R-o-18.

Table 1
Primers designed to amplify *BrFT1*and *BrFT2* from *Brassica rapa*.

Gene name	Primer name	Primer sequence (5′–3′)
BrFT1	*BrFT1*F	TCAACTCCCAAGACACTA
	*BrFT1*R	TATCACCGTTCATTGCTC
	BrFT1^{e2-4}F	TCCTAGCAATCCTCACCTCC
	BrFT1^{e2-4}R	GAGTGTTGAATTGTTGGCGC
BrFT2	*BrFT2*F	AGGGTTGGTTCAGAAGAT
	*BrFT2*R	TTAGGGCATGTAAAGTCA
	*BrFT2*F1	GTAGAGGGTTGGTTCAGA
	*BrFT2*R1	TTTGGGCTTTGTTAGGAG
	*BrFT2*F2	GTGGATCCAGATGTTCCAA
	*BrFT2*R2	CAATACCAGCACGAGACGA
	*BrFT2*F3	TCCCTGCGACAACTGGAAC
	*BrFT2*R3	AAGTCAACTGATTAAACGAA
	*BrFT2*F4	TATTGTTAAGTATGCATGCA
	*BrFT2*R4	TGGTTCTATCAACCGCATAT
	*FT2*intron2R1	TTACGCTGTTTTCTTGTAGTGTAAC
	*FT2*intron2R2	GAATATACCGATGAAATACC
	BrFT2^{e3-4}F	GGTGACTGATATCCCTGCGA
	BrFT2^{e3-4}R	TTGTATAGCGCGGCAAACTC

products were cloned into pEASY-T1 vectors (TransGen Biotech, http://www.transgen.com) for sequencing. *Escherichia coli* strain Trans-T1 (TransGen Biotech, http://www.transgen.com) was heat shock-transformed with these constructs, and positive colonies were selected using kanamycin media (50 mg/L). Plasmid DNA was isolated and sequenced using an ABI3730XL DNA analyzer (PerkinElmer, USA). Sequence alignment and analysis were conducted using MegAlign of DNASTAR (http://www.dnastar.com).

2.5. Total RNA isolation, cDNA synthesis, and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from young leaves of one-month-old plants grown in July 2015 using the TRIzol Reagent (Sangon Biotech, Shanghai, China), following the manufacturer's instructions. Total RNA was treated using RNase-free DNase I (NEB, Beijing, China) at 37 °C for 30 min to remove genomic DNA contamination. RNA integrity was verified by conducting 1% agarose gelelectrophoresis. First-strand cDNA synthesis was performed using 1 μg of total RNA in a final reaction volume of 20 μL using a TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China), following the manufacturer's protocol.

Specific primers designed for *BrFT1* (Br*FT1*$^{e2-}$ F/R) and *BrFT2* (Br*FT2*$^{e3-4}$F/R) (Table 1) were used for qRT-PCR. The cDNA was then used as a template in a 10-μL reaction using the F-416L DyNAmo^{TM} Color Flash SYBR Green qPCR kit (Thermo Scientific, Waltham, MA, USA) to perform the subsequent reactions. Real-time PCR reactions were performed in a 384-well plate with an Applied Biosystems ABI Prism 7900 Sequence Detection System (Applied Biosystems). All gene expression analyses were performed with three independent biological replicates. Two independent technical replicates were performed for each sample. The thermal cycling conditions consisted of the following: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s, and at 64 °C for 1 min. The samples were initially normalized to a selected internal control gene (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*), and

the relative gene expression levels were determined using the $2^{-\Delta Ct}$ method [37].

2.6. Statistical analysis

ANOVA and analysis of association were tested by one-way ANOVA and one-tailed Pearson correlation, respectively, in IBM SPSS Statistics for Windows, Version 20.0 (IBM, Armonk, NY, USA). ANOVA was performed using marker genotype as a factor.

3. Results

3.1. Flowering time variation among RILs

Flowering time was evaluated in the two growing seasons, spring (May and June) and autumn (September to December), when *B. rapa* crops are commonly cultivated. A wide variation in flowering time was observed among the 139 and 136 RILs grown in spring 2011 and autumn 2012, respectively. In general, RILs flowered earlier when they were grown in spring than in autumn (*t*-test, $P = 1.2 \times 10^{-13}$), thus illustrating the large influence of environment on flowering time. In spring 2011, two RILs had not produced flower buds by the end of the experiment (62 days after sowing), whereas in autumn 2012, six RILs had not initiated flowering by the time the experiment was terminated (95 days after sowing). The earliest flowering lines (H5, H25, and H77) occurred at 21 DTF in spring 2011, whereas the lines H55 and H96 showed earliest flowering at 29 DTF in autumn 2012. Continuous distribution of flowering time was observed in both testing environments (Fig. 2). In spring 2011, 129 lines flowered between 30 and 50 DTF, but only 81 of these 129 lines flowered within this range in autumn 2012; the remaining 48 lines took more than 50 days to flower when grown in autumn. Transgression towards both early and late flowering within the population was observed in both growing seasons. The parental line L58 was only slightly affected by growing season (35 DTF in spring, 39 DTF in autumn), whereas the flowering time of the parental line R-o-18 was significantly delayed in autumn (39 DTF in spring, 59 DTF in autumn; *t*-test, $P < 0.005$).

3.2. Genotyping and construction of the linkage map for RIL population

The parental lines L58 and R-o-18 were re-sequenced on an Illumina GA II platform. 73 and 53 nt pair-end (PE) reads were generated for 300-bp insert libraries constructed for L58 and R-o-18, respectively. Approximately 14.4 million and 21.4 million high-quality PE reads (average quality: ≥20) were generated for L58 and R-o-18, respectively. Using the method of Liu et al. [31], 18,479 short (<5 bp) InDel polymorphisms were detected between L58 and R-o-18, among which, 445 short InDels were converted to PCR-based markers. Approximately 202 of the 445 InDels (45.4%) were found to have identifiable polymorphisms between L58 and R-o-18 by using polyacrylamide gel electrophoresis. In addition, 197 of 1110 previously developed InDel markers [31,32] were determined to be polymorphic between L58 and R-o-18, of which 94 InDels had been mapped to the reference map used for the *B. rapa* pseudochromosome assembly [17]. In total, 386 polymorphic InDel markers were genotyped for the 149 RILs to construct a linkage map (Table S1). The linkage map covered 1071.5 cM, with an average distance of 3.0 cM between the ordered markers (Fig. 3).

3.3. Detection of flowering time QTLs and candidate genes for the QTLs

Five QTLs for flowering time were detected (Table 2), namely, three in spring 2011, and two in autumn 2012. The three QTLs detected in spring 2011 were located on chromosomes A07 (Flt1), A02 (Flt2), and A04 (Flt3); these explained 38% of flowering time variation within the population. The two QTLs (Flt4 and Flt5) detected in autumn 2012 co-located with Flt1 and Flt2, respectively, and explained 43% of flowering time variation.

Among the three spring 2011 QTLs, Flt1 was the most significant, explaining 19.4% of phenotypic variation. This QTL showed a positive additive effect, indicating that the L58 allele at this locus accelerated flowering. Flt4, which co-located with Flt1, explained an even higher of the phenotypic variation (27.6%) in the autumn 2012 experiment. The two co-locating A02 QTLs, Flt2 and Flt5, explained 12.9% and 16.5% of flowering time variation in spring 2011 and autumn 2012, respectively. Both mapped to 12.6 cM with a peak position, which is the same location of a *BrFLC2* flowering time gene specific InDel marker [23]. The parental line R-o-18 contains a 57-bp deletion mutation in *BrFLC2*, encompassing part of the fourth exon and the fourth intron [23]. This leads to alternative transcripts compared to that generated by the wild-type alleles, most likely rendering this allele non-functional. This is consistent with the negative additive effects of QTL identified at this locus in the two growing seasons; therefore,the L58 allele at this locus delayed flowering. In addition to the two common QTLs identified in both experiments, a QTL with a relatively low phenotypic effect was mapped to A04 (Flt3) and explained 8.2% of flowering time variations in spring 2011.

The peak marker associated with Flt1 and Flt4 was BrID11615. This marker was localized 28 kb upstream of the gene *Bra004117* (Fig. 4). Synteny analysis comparing *B. rapa* and *A. thaliana* [38] revealed that this gene was an orthologue of the *A. thaliana FT* gene. This gene was most proximal to BrID11615 that harbored an *A. thaliana* orthologue involved in flowering time control (Table S2). Two syntenic orthologues of *FT* were detected in *B. rapa*, and these are located on A02 (Bra022475) and A07 (*Bra004117*), respectively. The two genes are now named as *BrFT1* (A02) and *BrFT2* (A07). Wang et al. [39] reported six *FT* paralogous genes in *Brassica napus* (AACC) following screening of a BAC library. Amongst these, three mapped to chromosomes of the A genome and three to chromosomes of the C genome. For the three copies in the A genome, one that mapped to chromosome BnA2 was consistent with the *FT* orthologue identified on A02. However, the two copies detected on BnA7 were located adjacent to each other at the same locus and were named BnA7.FT.a and BnA7.FT.b, respectively. In *A. thaliana*, *FT* is located on ancestral block E of chromosome 1, which corresponds to three duplicated E blocks in the *B. rapa* genome [17]. Comparative genome analysis between *B. rapa* and *A. thaliana* [38], and the distribution of ancestral genome blocks in the *B. rapa* genome [40], revealed two E blocks linked end-to-end on chromosome A07; these belonged to the LF and MF2 sub-genomes, respectively. Therefore, if both replicated copies were retained during the gene fractionation process, then there should be two *FT* paralogues on A07, as reported by Wang et al. [39]. Investigation of the corresponding region where *FT* is located revealed that the syntenic copy in MF2 was lost (Fig. 5). The corresponding region of the *B. napus* genome [41] was analyzed to further confirm that only one *FT* orthologue was present on A07 (Table S2). Since FT paralogue was lost from the adjacent E block of MF2 sub-genome, the *BrFT2* gene of the LF subgenome located close to the peak marker of Flt1 and Flt4 was considered as the possible causal gene for these QTLs on A07.

3.4. Sequence polymorphisms of the BrFT2 gene between L58 and R-o-18

To identify sequence variations in the *BrFT2* gene between the two parental lines, L58 and R-o-18, primers were designed to amplify the *BrFT2* sequence (Figs. 1 and S1, Table 1). A 2499-bp

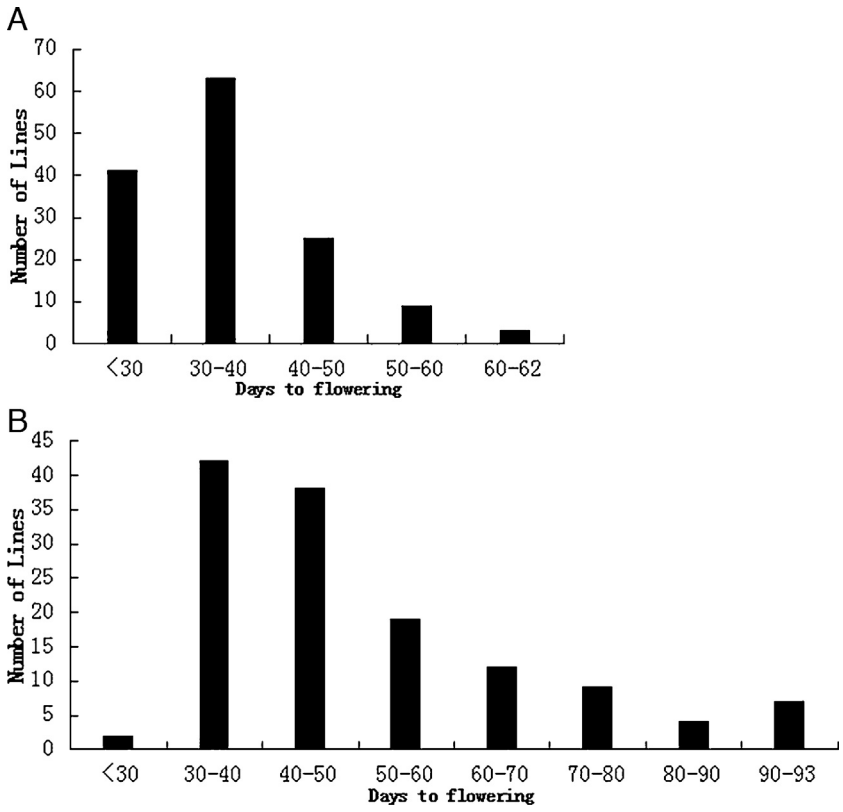

Fig. 2. Frequency distribution of the flowering-time phenotype in the RILs population.
(A) Distribution of flowering-time in the *Brassica rapa* RIL population in spring 2011. (B) Distribution of flowering-time in the *B. rapa* RIL population in autumn 2012. 62NF indicates plants had not developed flower buds at 62 days after sowing; 95NF indicates plants had not developed flower buds at 95 days after sowing. L, indicates parent line L58; R, indicates parent line R-o-18.

Table 2
Flowering time QTLs detected in the *Brassica rapa* L58× R-o-18 RIL population.

Year	QTL	Linkage group	LOD	Peak position (cM)	% EXP	Additive effect	Total (% EXP)
2011	Flt1	A07	8.3	80.9	19.4	4.2	38
(Spring)	Flt2	A02	5.7	12.6	12.9	-3.4	
	Flt3	A04	3.7	85.5	8.2	2.8	
2012	Flt4	A07	11.2	80.9	27.6	8.9	43
(Autumn)	Flt5	A02	7.2	12.6	16.5	-6.8	

fragment was amplified using L58 and primers located in the 5′- and 3′-UTRs of *BrFT2*; however, no product was amplified using R-o-18. Additional primer sets were designed to amplify the genomic sequence of *BrFT2* of R-o-18 (Fig. 1). DNA fragments were amplified using the primer pairs, with the exception of those encompassing the region between 259 bp and 640 bp of intron 2. This finding suggested that there might be a large insertion or structural variation within this region, leading to failed PCR amplification.

To confirm the presence of an insertion or structural variation in the R-o-18 allele of the *BrFT2* gene, the re-sequencing data of four *B. rapa* accessions were analyzed. These comprised PE reads from L58 (5×), R-o-18 (5×), as well as the re-sequencing data of VT117 (ssp. *rapa*, 20×) and L144 (ssp. *tricolaris*), 20× published by Cheng et al. [42]. Reads were mapped to the *BrFT2* gene region, and revealed that in R-o-18 and L144, there were no PE reads encompassing the 259–640 bp region of intron 2 were detected, whereas the other two accessions showed PE reads (Fig. 6). This result supported the notion that the *BrFT2* gene harbored a large insertion or structural variation in the R-o-18 allele.

To determine the exact position of the insertion or structural variation, PE reads of R-o-18 were mapped to the reference *B. rapa* genome using SOAP2 [43]. Ten PE reads with one end aligned to the 5′ flanking region of the failed amplified region of intron 2 were generated, whereas the other end did not. Unmapped ends were subsequently aligned against the reference genome sequence. Five of these aligned to transposon sequences, TE081156:LTR and TE398620:Unknown (BRAD, http://brassicadb.org). Because libraries with 300-bp insertions were used in the generation of the re-sequencing data, assembly of the inserted fragment could not be performed using reads from either the L144 or R-o-18. Using the primer *FT2*intron2R1 that was designed based on the reads aligned to TE081156:LTR in combination with the forward primer *BrFT2*F2 located in the second exon of *BrFT2*, a 519-bp fragment was amplified from R-o-18. Sequencing of the PCR product revealed that the transposon inserted at the 464 bp position of intron 2 (see Fig. S1). However, no PCR product was generated using primer pair of *BrFT2*F2/*FT2* intron 2R2 based on the reads aligned to TE398620:Unknown.

3.5. Correlation between sequence variations in the candidate gene BrFT2 and flowering time variation in the RIL population

Using the transposon insertion in intron 2 of the *BrFT2* gene as a molecular marker, the association between the allelic variation

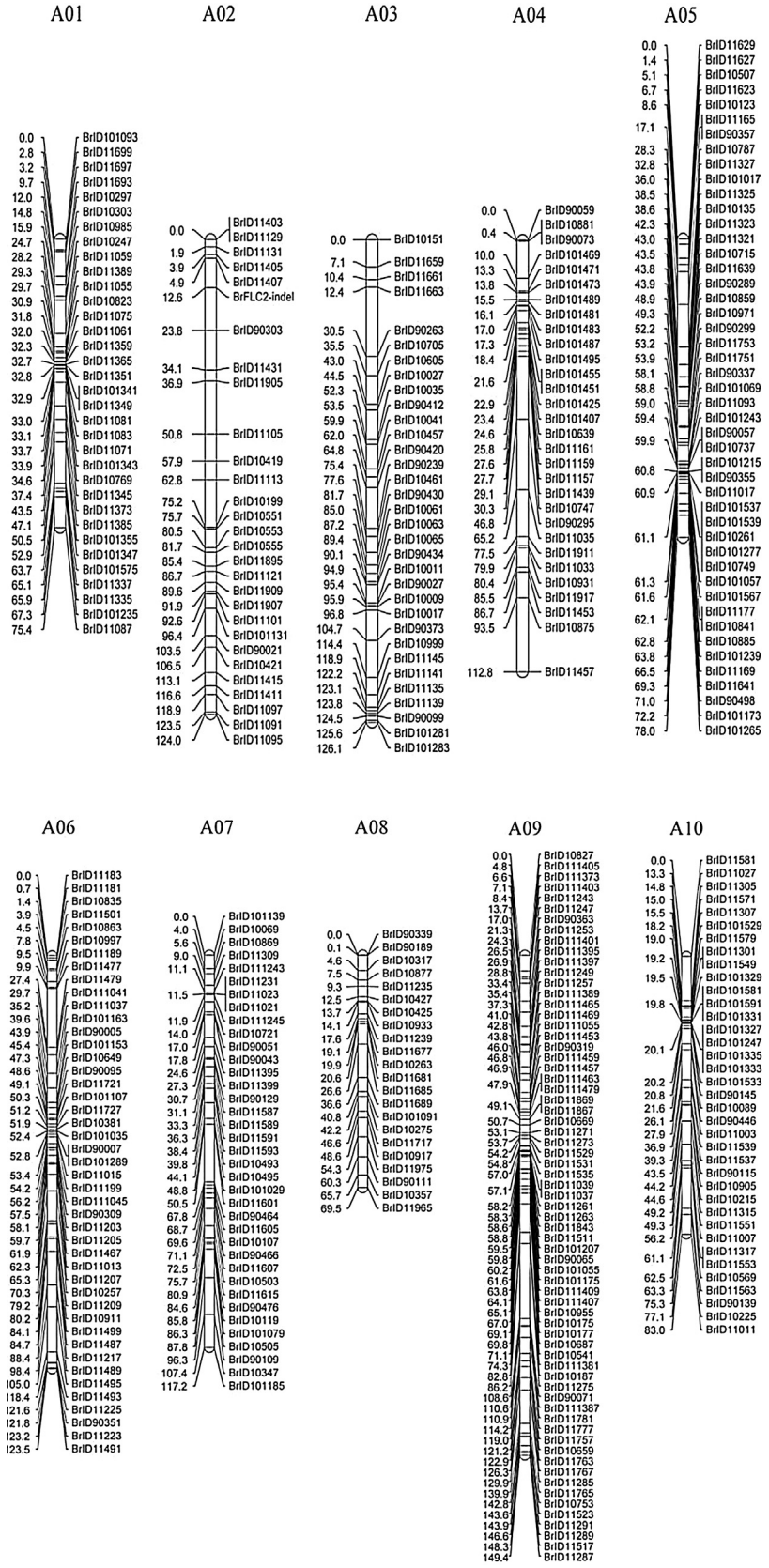

Fig. 3. Genetic linkage map of the *Brassica rapa* L58× R-o-18 RIL population.
The positions of 386 InDel markers distributed over 10 linkage groups corresponding to the 10 *B. rapa* chromosomes.

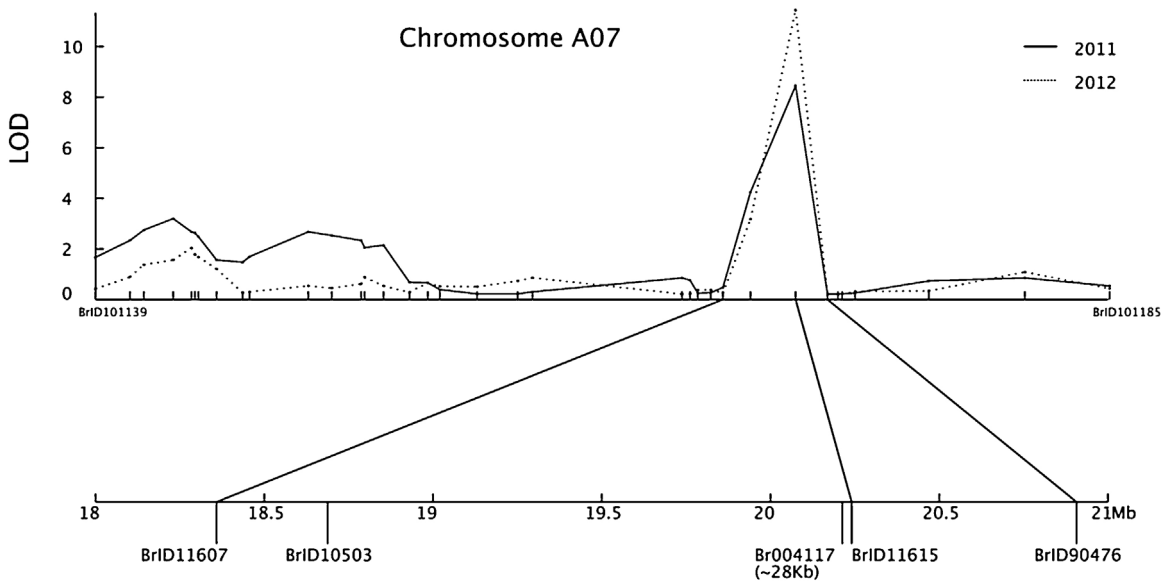

Fig. 4. Flowering time QTL detected on chromosome A07 in the *Brassica rapa* RIL population, and the genome region corresponding to the LOD peak.
The solid line indicates QTL profiling of data from spring 2011. The dash line indicates data from autumn 2012. Markers within the region and the candidate gene are indicated at the bottom.

of candidate gene *BrFT2* and flowering time was re-analyzed. A significant correlation between the R-o-18 *BrFT2* marker with flowering time variation was detected (spring 2011: $r = 0.253$, $P < 0.01$; autumn 2012: $r = 0.420$, $P < 0.001$). The 78 RILs homozygous for wild-type *BrFT2* flowered 4.9 days earlier than RILs carrying the mutant R-o-18 *BrFT2* allele in spring 2011, whereas flowering time differed by 14.7 days between the two groups in autumn 2012 (Table 3). When the *BrFLC2* allele was taken into account, a correlation between flowering time of RILs with different *BrFT2* alleles and *BrFLC2* genotypes was also observed (Table 3). This finding was consistent with the location of the *FT* downstream of the *FLC* in the flowering time regulation pathway.

Table 3
Average flowering time in the RIL populations according to the different genotypes of *BrFT2* and *BrFLC2*.

Year	*BrFT2*/*B rFLC2* genotype	No. of lines	Days to flowering (DTF)
2011 Spring	*BrFT2BrFLC2*	36	34.8 ± 9.8 a
	Brft2BrFLC2	24	43.5 ± 9.9 b
	BrFT2Brflc2	35	32.2 ± 6.1 a
	Brft2Brflc2	29	33.4 ± 8.5 a
2012 Autumn	*BrFT2BrFLC2*	38	48.5 ± 16.5 b
	Brft2BrFLC2	26	68.8 ± 17.8 c
	BrFT2Brflc2	36	38.9 ± 6.2 a
	Brft2Brflc2	29	47.6 ± 13.0 b

Note: DTF data are presented as means ± standard error. Significant differences ($P \leq 0.05$) between lines carrying different alleles of the *BrFT2* and *BrFLC2* genes are indicated by different letters.

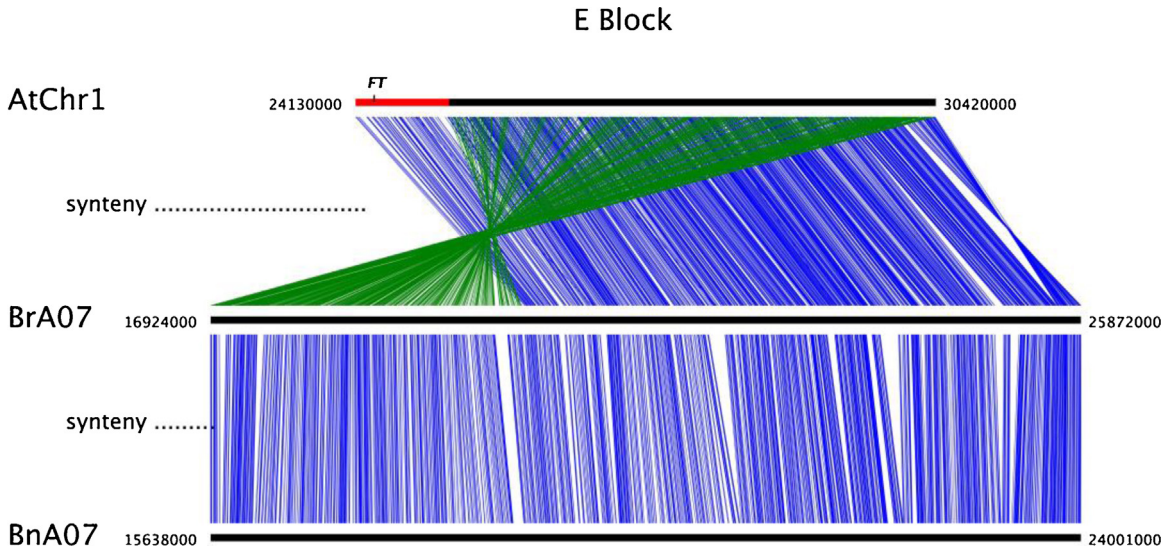

Fig. 5. Collinearity between ancestral E blocks from *A. thaliana* chromosome 1, *B. rapa* chromosome A07, and *B. napus* chromosome Bn07. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The red bar indicates the fragment lost in the MF2 subgenome of *B. rapa* and in the BrMF2 subgenome of *B. napus*.

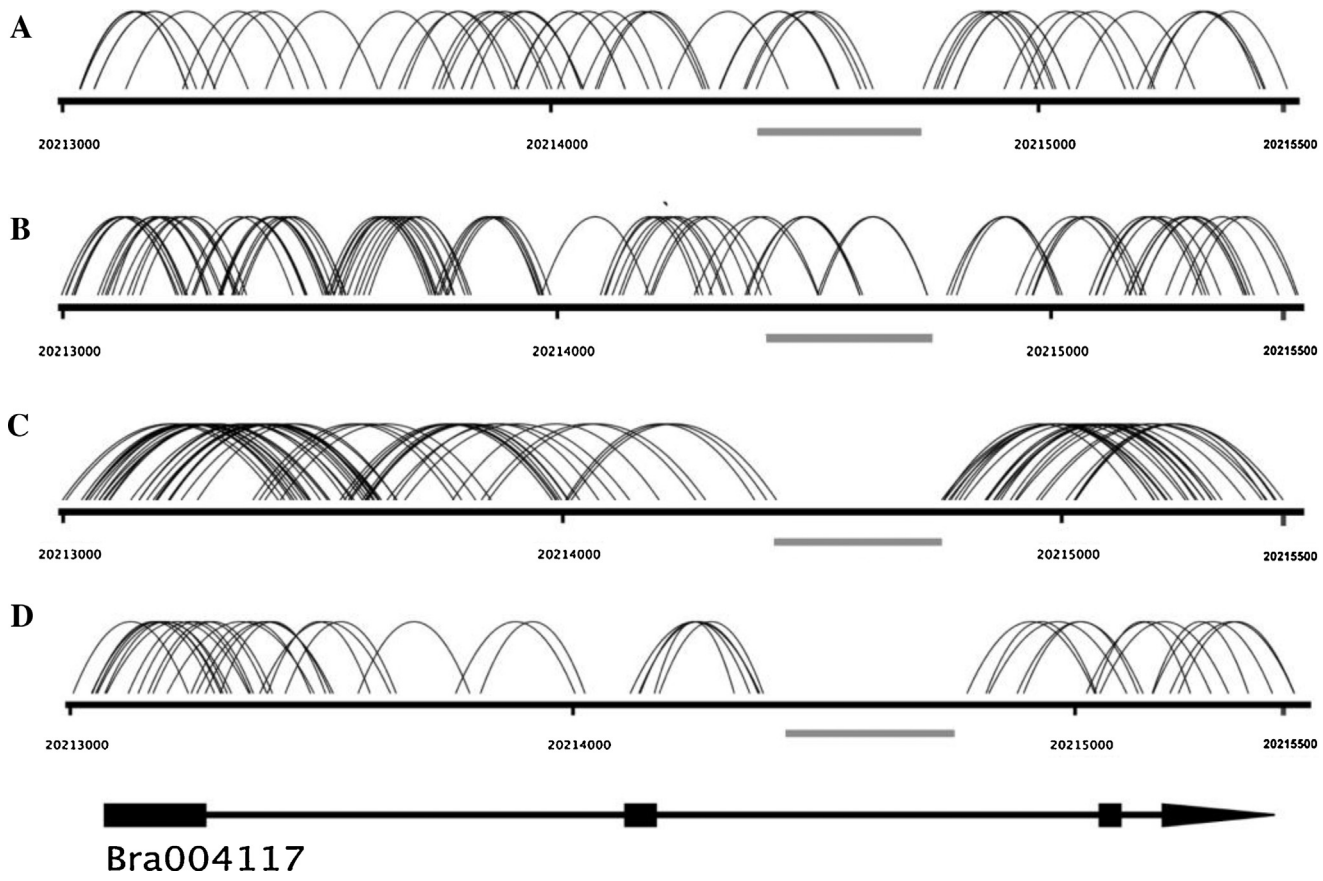

Fig. 6. Use of paired-end reads alignment to identify the insertion in intron 2 of the *BrFT2* gene.
The plot shows the paired-end reads mapping to the region flanking *BrFT2* on chromosome A07. The arcs indicate paired-end reads aligned to the reference sequences; the grey bars indicate the region that could not be amplified in parental line R-o-18; the solid boxes indicate exons; the lines between the boxes indicate introns; the arrow indicates the transcriptional orientation. (A) L58 (ssp. *parachinensis*). (B) VT-117 (ssp. *rapa*). (C) L144 (ssp. *tricolaris*). (D) R-o-18 (ssp. *tricolaris*).

3.6. Failed transcription of the BrFT2 *gene is due to the transposon insertion*

To further confirm that the large insertion in the *BrFT2* gene was responsible for the delayed flowering in R-o-18, quantitative real time RT-PCR was performed on the *BrFT1* and *BrFT2* genes to determine their transcription levels in the two parental lines (grown during June and July 2015). Gene-specific primers were designed to amplify the exons 2–4 of the *BrFT1* and exons 3–4 of the *BrFT2* gene (Fig. 1). The results revealed that the *BrFT1* and *BrFT2* genes were transcribed at comparable levels in one parental line, L58. In R-o-18, the transcript level of *BrFT1* was slightly higher than that in L58, whereas *BrFT2* was minimally expressed (Fig. 7). Therefore, the transposon insertion of *BrFT2* into the R-o-18 allele likely prevented correct transcription, thereby rendering a non-functional allele.

4. Discussion

Both *B. rapa* and *A. thaliana* are LD plant species. Earlier flowering occurred when the *B. rapa* RILs were grown in long day length (LD) as opposed to short day length (SD) seasons; this finding demonstrated the impact of photoperiod on the regulation of flowering time in *B. rapa*. In *A. thaliana*, the FT protein is recognized as a major component of the 'florigen', and has a central position in mediating the onset of flowering [1,5,44]. Various factors, including photoperiod, temperature, plant age, and gibberellins converge to regulate *FT* expression in the control of flowering time. Higher expression of *FT* corresponds to earlier flowering. Therefore, introducing a mutated *FT* allele that would block internal and external signals could be an efficient way to delay plant flowering. This strategy is difficult to achieve in *Brassica* species because the whole-genome triplication event gave rise to multiple copies of flowering time genes; according to BRAD, there are four *FLC*, three *VRN1*, and two *FT* copies in the *B. rapa* genome. It is difficult to predict whether delayed flowering can be achieved by interfering with the expression of only one of the paralogues. In the present study, a QTL on chromosome A07 was detected in different growing seasons. This co-localized with a syntenic ortholog of *A. thaliana FT*, which was named as *BrFT2*. The RILs carrying loss-of-function allele *Brft2* showed delayed flowering regardless of growing season when an active *BrFLC2* allele was present. The delayed flowering caused by a loss-of-function allele of *BrFT2* indicated that, at least to some extent, the two paralogous *FTs* had diversified functions, and that it is feasible to regulate flowering time by interfering with only one *FT* paralogue.

Although plants carrying the loss-of-function allele of *BrFT2* showed delayed flowering time in LD and SD seasons, the delay was 66.7% lower in plants grown under LDs than that in SDs. This contrasts to the phenotype observed in *A. thaliana ft* mutants, which showed a late flowering phenotype under LD conditions, with flowering time only slightly affected under SD conditions [45]. This difference could be due to the functional redundancy of the two *FT* paralogues in *B. rapa*, which are likely to be partially redundant, given that the mutation in *BrFT2* resulted in delayed flowering. Furthermore, we cannot exclude the possibility that there is functional diversification between *BrFT1* and *BrFT2*, wherein BrFT2 controls flowering under SDs. In *A. thaliana*, *TWIN SISTER OF FT* (*TSF*) is the

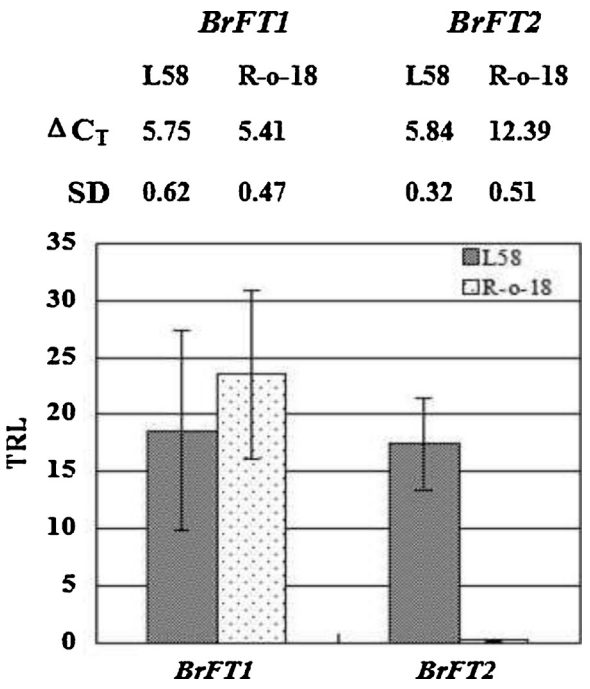

Fig. 7. Transcript levels of *BrFT1* and *BrFT2* in *Brassica rapa* accessions, L58 and R-o-18.
Transcript levels were assessed by quantitative real-time RT-PCR in young leaves of one-month-old plants relative to *GAPDH* as a constitutively expressed control gene. Values are mean relative transcript levels calculated from arithmetic means of ΔC_T values of three biological replicates. Error bars represent SD for three replicates. RTL: relative transcript level. RTLs were calculated as follows: $RTL = 1000 \times 2^{-\Delta CT}$.

closest homolog of *FT*, and acts as a floral pathway integrator redundantly with *FT* [46,47]. The *tsf* mutant shows no obvious difference in flowering time to that of wild-type (WT) plants with LDs, but has delayed flowering compared to WT plants with SDs [47]. This is consistent with that observed for *BrFT2*. No syntenic orthologous gene of *AtTSF* was detected in the *B. rapa* genome (http://brassicadb.org/brad), which suggests that *BrFT2* might have phenocopied the function of *AtTSF* in *B. rapa*. To clarify the function of the two *FT* paralogues in *B. rapa*, it will be necessary to perform functional complementation assays using *FT* mutants of *A. thaliana*, as well as *B. rapa,* using these two genes, given that *B. rapa* mutants with the same genetic background are currently available (http://revgenuk.jic.ac.uk/).

In addition to the *BrFT2* locus, a QTL was detected in the region where *BrFLC2* was located, despite the RIL population being grown in a vernalization-free environment in both growing seasons. The explained phenotypic variation at this QTL varied between 12.9% in the spring and 16.5% in the autumn; this is much lower than the observed 63.8% variation without vernalization, and the reported 30.9% using 18 days of vernalization, as reported by Zhao et al. [26]. The difference between the two studies might be due to differences in the flowering behavior of the parental lines used in the construction of the population. Both parents of the RILs used in the present study were rapid cycling, without a vernalization requirement, whereas one of the parents used in the study conducted by Zhao et al. [26] was a vernalization-requiring accession. Several previously reported QTL mapping studies on flowering time in *B. rapa* only identified loci where *FLC* genes were located [22,25,26]; it is therefore assumed that the strong effect imposed by *FLC* on flowering time variation masked the effects of other pathways or genes in the same pathway. In contrast to the notion that the segregating population constructed by parents shows obvious phenotypic differences, this observation suggests that to successfully dissect the genetic control of flowering time, populations are also required to be constructed by crossing parents with similar performances of the target trait.

Although the contribution to flowering time variation by *BrFLC2* locus was lower than the *BrFT2* locus in the present study, the findings indicated that *BrFLC2* plays an important role in *BrFT2* function. When loss-of-function allele *Brflc2* present, the lines flowered early regardless of the existence of loss-of-function allele *Brft2* or functional allele *BrFT2*. This observation was consistent with the fact that *FLC* represses the expression of *FT*. Furthermore, the transcript level of *BrFT1* was slightly lower in the parental line L58 than that in R-o-18. These results indicated that *BrFLC2* might repress the transcription of both *FT* paralogs, but not only *BrFT2*.

Using the same population, Bagheri et al. [29] identified five QTLs for flowering time, one of which was located close to the position of the QTL identified on A07 in the present study. Because only a few common markers between the two linkage maps have been identified, it is difficult to determine whether the QTL identified in the two studies are one and the same. However, if they were the same locus, then this means it is a robust QTL, and it could therefore be an excellent preferred target for the improvement of premature bolting in *B. rapa* crop cultivation.

The development of new sequencing technology and the associated decrease in sequencing cost, has led to an increase in the number of genomes being sequenced. This facilitates fine mapping and cloning of candidate genes underlying desired QTLs. In the present study, the availability of the whole *B. rapa* genome sequence [17], together with comparative alignment with the closely related model plant species, *A. thaliana* [20,36], allowed the identification of *BrFT2* as the causal candidate gene underlying the major QTL.

The present work identified a mutation in an *FT* gene that is involved in flowering time regulation in *B. rapa*. Further gene function analysis of *BrFT1* and *BrFT2* is required to dissect the diversification of the two *FT* paralogues in *B. rapa.* Considering the important role *FT* plays as '*florigen*' in the regulation of flowering time, knowledge about these *FT* genes and detected loss-of function mutated alleles in *B. rapa* will be useful for breeding improvement of late-bolting *B. rapa* crops.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.10.007.

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