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

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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

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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