Fine Mapping and Analysis of Candidate Genes for qFT7.1, a Major Quantitative Trait Locus Controlling Flowering Time in *Brassica Rapa* L

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Research Article

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Abstract

In *Brassica rapa*, flowering time (FT) is an important agronomic trait that affects the yield, quality, and adaption. FT a complicated trait that is regulated by many genes and is affected greatly by the environment. In this study, a chromosome segment substitution line (CSSL), CSSL16, was selected that showed later flowering than the recurrent parent, rapid-cycling inbred line of *B. rapa* (RcBr). Using Bulked Segregant RNA sequencing, we identified a late flowering quantitative trait locus (QTL), designated as *qFT7.1*, on chromosome A07 based on a secondary-F_2 population derived from the cross between CSSL16 and RcBr. *qFT7.1* was further validated by conventional QTL mapping. This QTL explained 39.9% (logarithm of odds = 32.2) of the phenotypic variations and was fine mapped to a 56.4-kb interval using recombinant analysis. Expression analysis suggests that *BraA07g018240.3C*, which is homologous with *ATC* (encoding *Arabidopsis thaliana CENTRORADIALIS* homologue), a gene for delayed flowering in *Arabidopsis* as the most promising candidate gene. Sequence analysis demonstrated that two synonymous mutations existed in the coding region and numerous bases replacements existed in promoter region between *BraA07g018240.3C* from CSSL16 and RcBr. The results will increase our knowledge related to the molecular mechanism of late flowering in *B. rapa*, and lay a solid foundation for the breeding of late bolting in *B. rapa*.

Key Message

qFT7.1, a major QTL for flowering time in *Brassica rapa* was fine-mapped to chromosome A07 in a 56.4-kb interval in which the most likely candidate gene is *BraA07g018240.3C*.

Introduction

The economically important crop, *Brassica rapa*, has long been cultivated worldwide, mainly as a vegetable foodstuff, such as Chinese cabbage and Pak-choi, and to a lesser extent for the production of fodder and oilseed, such as turnip rape and yellow sarson (Carpio et al. 2011). Among agronomic traits in *B. rapa*, flowering time (FT) is important because it affects the yield of seeds and the harvested crop's commercial quality (Wu et al. 2012), thus determining their growing season and cultivation area (Xiao et al. 2019). The regulation of FT is complex, involving multiple genes (quantitative trait loci (QTLs)) and is markedly affected by environmental conditions, making it a challenge to identify linked markers or related genes for marker assisted selection (MAS)-based breeding (Liu et al. 2016).

Many QTLs related to FT in *B. rapa* have been identified in the last 30 years, mainly based on a wide range of bi-parental mapping populations or natural populations (Teutonico and Osborn 1995; Osborn et al. 1997; Ajisaka et al. 2001; Schranz et al. 2002; Nishioka et al. 2005; Yang et al. 2007; Lou et al. 2007; Li et al. 2009a; Yuan et al. 2009; Zhao et al. 2010; Lou et al. 2011, Kakizaki et al. 2011; Xiao et al. 2013; Li et al. 2015; Zhang et al. 2015; Liu et al. 2016; Xi et al. 2018; Wang et al. 2018b; Xiao et al. 2019; Fu et al. 2020; Kaur et al. 2021). The majority of these populations are traditional primary populations, including F_2, BC_1, doubled haploid (DH), and recombinant inbred lines (RILs). However, these populations are only

useful to detect QTLs with relatively large effects, because the segregants often have complicated backgrounds due to the presence of large parent-derived chromosomal fragments, thus QTLs with minor effects might be missed. By contrast, a wider range of QTLs can be identified using advanced mapping populations, including nearisogenic lines (NILs) and chromosome segment substitution lines (CSSLs) (Nadean and Frankel. 2000). Moreover, secondary F_2 or F_3 populations can be produced by from backcrossing a target NIL or CSSL with the recurrent parent, making them more suitable for fine mapping and positional cloning of a target QTL (Yano. 2001). Over 75 CSSL libraries in 17 major crops have been constructed in the last three decades, which have made significant contributions to QTL characterization (Balakrishnan et al. 2019), despite the development of these population being labor and time intensive. However, currently, only few CSSLs are available for *B. rapa* (Li et al. 2015; Wang et al. 2018b). Most of the above mentioned QTLs identified in primary populations have not yet to be fine mapped, mainly because of a lack of optimal genetic material, and very few studies resulted in the actual cloning of the gene responsible for flowering in *B. rapa*. To the best of our knowledge, to date, only a few genes, such as *BrVIN3.1, BrFLC1*, *BrFLC2* (Su et al. 2018; Jeong et al. 2019) and *BraELF6* (Li et al. 2019) have been successfully cloned and subsequently confirmed by transformation in *B. rapa* and in *Arabidopsis thaliana*, respectively.

NILS and CSSLs have been demonstrated as effective resources to validate QTLs and can be used to create fine-mapping populations (Fletcher et al. 2013). CSSLs (or NILs) lines containing a single (or several) genomic introgression from the donor parent in a different, but homogenous, genomic background. Thus, CSSLs eradicate the 'noisy' genetic background, permitting QTLs to be resolved as Mendelian factors. CSSLs and NILs have advantages in transcriptional analyses because interference from the genetic background is minimized, thus enhancing the accuracy and sensitivity of transcriptional analysis (Keurentjes et al. 2007). Transcriptional analysis (e.g., RNA sequencing (RNA-seq)) based on CSSLs (or NILs) has contributed to the rapid identification of candidate genes underlying QTLs, especially in species with large polyploid genomes, such as wheat (Barrero et al. 2015; Xiao et al. 2016; Habib et al. 2018; Yang et al. 2018; Jiang et al. 2019).

Recently, the identification and isolation of genes underpinning QTLs associated with agronomic traits in crops have been accelerated significantly because of the emergence of rapid and inexpensive next-generation sequencing (NGS)-based technologies combined with plant genetics (Nguyen et al. 2019). An example of such achievement is QTL-seq (Takagi et al. 2013), which combined with bulk-segregant analysis (BSA), can rapidly discover associated markers and candidate genes by sequencing the bulks and parents with extreme phenotypes from the segregating populations, using BSA based on DNA-and RNA-seq (Zou et al. 2016). Traditional QTL mapping integrated or combined with QTL-seq and RNA-seq is a highly efficient and accurate approach for QTL mapping and validation, which enables the identification of candidate genes associated with agronomic traits of interest, and has been widely utilized in diverse crops (Lu et al. 2014; Berenguer et al. 2015; Gelli et al. 2017; Li et al. 2017; Shu et al. 2018; Wang et al. 2018a; Liu et al. 2019; Park et al. 2019; Wen et al. 2019; Huang et al. 2021).

Previously, a set of CSSLs was developed by our group using rapid-cycling inbred line of *Brassica rapa* (RcBr) and the Chinese cabbage inbred line 08A061 as the recipient and donor parents, respectively (Wang et al. 2018b). Among the developed CSSLs, CSSL16 showed significantly later flowering than the recurrent parent, RcBr. To map and identify the candidate gene(s) responsible for this late flowering, we developed a secondary F_2 population derived from a cross between CSSL16 and RcBr. Bulked Segregant RNA-Seq (BSR-Seq) identified a QTL, *qFT7.1*, which was validated using conventional QTL mapping. Ultimately, recombinant analysis narrowed down *qFT7.1* to a 56.4-kb interval on chromosome A07, allowing the candidate genes to be identified. Our findings represent a benchmark to further determine the molecular mechanism of late flowering in *B. rapa.*

Materials And Methods

Plant materials and trait measurement

Our laboratory previous constructed a set of CSSLs populations from a cross between RcBr as the recipient parent, which is an extremely early-flowering and vernalization independent inbred line, and 08A061 as the donor parent, which is a very late-flowering and vernalization dependent Chinese cabbage inbred line (Wang et al. 2018b). In the CSSL population, one line, CSSL16, with a late flowering phenotype, was chosen for backcrossing with the recurrent parent, RcBr. The secondary F_2 CSSL16/RcBr population was subsequently constructed using self-pollination.

The phenotypic analyses were carried out at the Experiment Station of Shenyang Agriculture University, Shenyang, China (41.8°N, 123.4°E). Four flowering indices were assessed to calculate the phenotypic scores of the individuals: The bolting index (BI), days to reach a 5/10-cm-high elongated floral stalk (DE5/DE10), and FT (Liu et al. 2016). CSSL16 and RcBr were evaluated under four environmental conditions (E1, E2, E3 and E4, Table S1). A secondary F_2 population consisted of 500 individuals was sown in the green house in September 2019, which were utilized for BSR-Seq and 300 individuals were used for conventional QTL analysis in March 2020. The progeny of the recombinant individuals screened from the secondary F_2 population (2200 individuals), along with parental controls, were grown in March 2021 for fine mapping of the identified QTL. All plants were sown directly into 10-cm pots without providing any extra vernalization, as described in our previous study (Wang et al. 2018b).

RNA isolation and extreme pool construction

For BSR-Seq analysis, we constructed two extreme pools, a L-pool (late-flowering pool) and an E-pool (early flowering pool), by mixing the same amounts of RNA from 25 late-flowering or 25 early-flowering plants, according to the phenotypic scores of 500 F_2 individuals recorded in the fall of 2019. About 50 days after sowing, the apex leaves of each pool were sampled and subjected to RNA. The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA. An Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1% agarose gel were used to assess the quantity and quality of the extracted

RNA. RNA (1 μg) with an RNA integrity number (RIN) > 7 was then processed for next generation sequencing library construction (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®, NEB, Ipswich, MA, USA).

BSR-seq

The prepared cDNA libraries were sequenced using the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) in the 2´150 bp paired-end (PE) configuration, after which the sequences were processed and analyzed by GENEWIZ (Suzhou, China). To remove technical sequences, including adapters, polymerase chain reaction (PCR) primers (or fragments thereof) and bases with quality lower than 20, Trimmomatic (v0.30) (Bolger et al. 2014) was used to generate high quality clean data. The clean data were mapped to the *B. rapa* reference genome V3.0 (BRAD, http://brassicadb.cn/#/Download/) using Hisat2 (Zhang et al. 2018a). Samtools v0.1.18 (Li et al. 2009b), with the command mpileup, and Bcftools v0.1.19 (Narasimhan et al. 2016) were used to carry out Single Nucleotide Variation (SNV) calling. The Euclidean distance (ED) value was calculated based an mpileup file which was generated using samtools v0.1.18 for BSR-seq.

According to the basic principle of the ED value, the occurrence frequency of the four bases A, T, C and G at the SNV site was statistically different in the population, and the corresponding base frequency of the two trait groups was calculated by distance. To eliminate the background noise, the ED value of each different SNV site was raised to the power of five, termed ED^5 (Su et al. 2016). All ED^5 values were sorted, and the different SNV sites corresponding to the top 1% of ED^5 values were screened, and then specific chromosome segments were located according to the distribution of the different SNV sites.

DNA extraction and marker development

The CTAB method, with minor modifications (Murray and Thompson 1980), was used to extract total DNA from the two parental lines and individuals of the secondary F_2 populations. The PCR reaction volume and amplification were same as those described previously (Wang et al., 2018b). The markers were designed using Primer Premier 5.0 software (Premier Biosoft, San Francisco, CA, USA) based on sequence variations of the target region identified by BSR-seq and whole-genome re-sequencing between RcBr and CSSL16. The primer information is shown in Table S2.

QTL analysis and fine mapping

The BSR-seq-identified QTL for FT was confirmed using classical QTL mapping assisted by polymorphic markers. The secondary F_2 populations utilized for conventional QTL analysis consisted of 300 individuals sown in March 2020. QTL mapping was performed using composite interval mapping (CIM) implemented in Windows QTL Cartographer 2.5 (Silva et al. 2012). The determined logarithm of odds (LOD) value for putative QTL declaration was determined after 1000 permutation tests at a significance level of $P < 0.05$ and a threshold of 3.0.

The progeny of recombinant individuals screened from a larger secondary F_2 population (2200 individuals) sown in a green house in March 2021 was used for fine mapping of the identified QTL. The means of the homozygous recombinant phenotype of the progeny were analyzed using Student's *t-test* in SPSS v17.0 (IBM Corp., Armonk, NY, USA), and compared with that of the control (RcBr) at a significance level of $P < 0.01$.

Whole-genome resequencing

Total DNA was extracted from young leaves. Its quality was determined using 0.8% agarose gel electrophoresis and it was quantified using an ultraviolet spectrophotometer. The Illumina NovaSeq platform was used for 2´150 bp paired-end sequencing. The raw data were was cleaned using Adapter Removal (version 2) (Schubert et al. 2016), and high quality reads were compared with the *Brassica rapa* V3.0 reference genome. Single nucleotide polymorphisms (SNPs) and InDels were detected by GATK software (Van et al. 2013) and analyzed using the ANNOVAR software (McKenna et al. 2010, Wang et al. 2010).

Candidate gene sequence analysis

The annotation information of genes in the candidate region was obtained from the *B. rapa* database (BRAD, http://brassicadb.cn/#/Download/) and The ArabidopsisInformation Resource (TAIR, http://www.arabidopsis.org/). The specific primers (Table S2) to amplify the full-length coding sequences and promoter sequences were designed using Primer 5.0 (Premier Biosoft). A Gel Extraction Kit (CWBIO, Beijing, China) was used to purify the PCR products, which were ligated into a pGEM-T Easy Vector (Promega, Madison, WI, USA), followed by sequencing at GENEWIZ. The sequences were aligned using the DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA) and the structure of the candidate gene was displayed using online software (http://gsds.cbi.pku.edu.cn/).

Quantitative real-time reverse transcription PCR (qRT-PCR) analysis of candidate gene expression

The expression level of the candidate gene was detected using qRT-PCR. Total RNA of RcBr and CSSL16 from roots, leaves, cotyledons, hypocotyls, stems, flowers, and the shoot apical meristem (SAM) were isolated using an RNA extraction kit (Aidlab Biotechnologies Co., Ltd., Beijing, China). The RNA was then reverse transcribed to cDNA. The cDNA was then used as the template for the qRT-PCR reaction (reaction volume: 25 μL, comprising 2 × Ultra SYBR Mixture, 2 μL of diluted cDNA, 1 μL of 0.2 μM forward and reverse primers, and 21 μL of RNase-free water). The reaction conditions were: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 °C for 1 min. This was followed by melting-curve analysis: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. The $2^{-\triangle\triangle Ct}$ method was used to analysis the relative expression level. Cycle threshold (Ct) values were shown as the means of three independent biological replicates. Each sample was analyzed as three independent technical replicates. QuantStudio™6 Flex Manager software (Livak and Schmittgen 2001) was used to analyze the data. Primer 5.0 was used to design gene-specific primers (shown in Table S2), with the Actin gene being used as the internal control (Huang et al. 2015).

Result

Genotypic and phenotypic characterization of RcBr and CSSL16

To detect the segment which had introgressed from 08A061 on ten chromosomes of CSSL16, the two parental lines, RcBr and CSSL16, were genotyped using whole-genome re-sequencing. A total of 67,507,156 and 64,540,134 high quality reads were detected in RcBr and CSSL16, and the clean data was compared to reference genome. This identified 2,999,421 SNPs and 627,670 InDels on the ten chromosomes between the two parental lines. According to the ED value calculation, the variation was mainly distributed in chromosome A02 (physical location 1,775,235–2,512,196 bp) and A07 (physical location 15,350,379–16,648,887 bp) (Fig.1). The total substituted segment derived from the donor parent, 08A061 was approximately 2.04 Mb, and the *B. rapa* whole genome size is about 353.14 Mb (*B. rapa* V3.0), and the background recovery rate was about 99.42% (351.10/353.14).

RcBr and CSSL16 showed a significant difference in FT under multiple environments (Fig.2a). Under E1 growth conditions, the FT of RcBr (75.47 ± 3.78) was earlier than CSSL16 (98.19 ± 2.07) by about 23 days, under E2 conditions, the FT of RcBr (52.26 ± 1.27) was earlier than CSSL16 (63.43 ± 1.28) by about 11 days, under E3 conditions, the FT of RcBr (44.21 ± 3.55) was earlier than CSSL16 (61.69 ± 3.88) by about 17 days, and under E4 conditions, the FT of RcBr (38.78 ± 2.78) was earlier than CSSL16 (51.55 ± 3.37) by about 13 days (Fig.2b). The two parental lines also showed significant differences in DE5, DE10, and BI under all four growth conditions (Table 1). In conclusion, RcBr and CSSL16 showed significant difference in all flowering-related traits.

Identification and validation of *qFT7.1*

Through BSR-seq analysis, we were able to map 45,425,180 and 41,697,006 clean reads to the *B. rapa* reference genome from the E-pool and L-pool, respectively. A total of 218,944 SNPs in the E-pool and 209,924 SNPs in the L-pool were identified. All ED^5 values were sorted, the top 1% of ED^5 values was used as the threshold and the different corresponding SNV sites were screened. The distribution of different SNV sites confirmed the candidate region. This candidate region of the QTL for FT was located on chromosome A07, starting at 15,486,952 and ending at 16,546,846, thus, the candidate interval covers about 1.06 Mb (Fig. 3). The candidate QTL underlying FT in this region was designated as *qFT7.1.*

To validate the QTL, *qFT7.1*, identified by BSR-seq analysis, we carried out conventional QTL analysis with 300 F_2 individuals in March 2020. A total of 13 polymorphic markers (Table S2) were screened from the difference interval (donor segment of 08A061), which was detected using whole genome re-sequencing (Chromosomes A02 and A07), and all polymorphic markers were used for classical QTL mapping. One QTL with a LOD value of 32.2, explaining 39.9% of the phenotypic variation, was found to control FT, and was located between marker InDel714 and InDel716, corresponding to a physical position of 15,539,588 bp to 16,499,043 bp on chromosome A07 (Fig. 4b), however, we could not detect any QTL on chromosome A02. Thus, the conventional QTL analysis confirmed the QTL *qFT7.1*, which was identified via BSR-seq analysis.

Fine mapping of *qFT7.1*

The candidate QTL, *qFT7.1*, was preliminary mapped to a 1.06 Mb candidate region on chromosome A07. A larger F_2 population consisting of 2200 individuals was used to refine the position of *qFT7.1*. Recombinant plants were screened with markers InDel723 and InDel716 and a total of 19 recombinant individuals were obtained. The homozygous recombinant progeny were divided into ten groups according to their genotypes. The mean value of the homozygous progeny (DE5, DE10 and FT) were compared with RcBr at *P<0.01* level.

Recombinants L1 and L2 both showed no difference with RcBr, while L3 and L4 had the opposite genotype to L1 and L2. Recombinants L3 and L4 both showed significant differences with RcBr, but no difference with CSSL16, thus these groups placed the QTL to a region upstream of markers InDel705 and InDel708. In the same way, the results for recombinants L5 and L6 demonstrated that the QTL was located upstream of markers InDel706 and InDel707, and the results for recombinant L7 placed the QTL in a region upstream of marker InDel702. Furthermore, recombinant L8 identified that the QTL was located upstream of marker InDel715, whereas recombinant L9 was significantly different from CSSL16 and similar to RcBr, which delimited the QTL to a region downstream of marker InDel714. Recombinant group L10 showed a significant difference with RcBr, thus group L10 placed the QTL in a region downstream of marker InDel723. By positioning of the recombinant groups, *qFT7.1* was finally narrowed down to a 56.4-kb interval between marker InDel714 and InDel715, and physical position of 15,539,588 bp to 15,595,959 bp on chromosome A07 (Fig. 4c).

Candidate gene annotation

According to the *B. rapa* reference genome database annotation, nine genes were annotated to the 56.4-kb region *(BraA07g018220.3C– BraA07g018300.3C*). The detailed information for the genes is shown in Supplementary Table 3. All the genes were compared with *Arabidopsis* homolog genes and analyzed for their function, we found that *BraA07g018240.3C* is homologous with *Arabidopsis* gene *At2g27550*, a key gene regulating FT. This gene was an *Arabidopsis CENTRORADIALIS* homologue (*ATC*) gene which belongs to *FLOWERING LOCUS T* (*FT*)family and encodes a protein similar to *TERMINAL FLOWER1* (*TFL1*), the overexpression of which leads to a similar phenotype as *TFL1*. The encoded protein from the identified gene might inhibit the expression of critical flowering genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*), acting in a non-cell autonomous manner to delay flowering (Huang et al. 2012, Zhu et al. 2020).

Expression analysis by qRT-PCR

The results indicated that *BraA07g018240.3C* was specifically expressed in the root and hypocotyl, and not in other tissues. The expression level showed significant differences in the hypocotyl, but not in the root between RcBr and CSSL16 (Fig. 5). The expression of the others eight genes was also detected in the root, stem, leaf, and flower, with *BraA07g018270.3C* and *BraA07g018300.3C* showing significantly different expression levels in flower (Fig. 6).

The signals of each flowering pathway were collected in the SAM, and were used together to determine the FT. *AP1* and *LFY* are both main inflorescence meristem genes, and play a central role in the flowering regulatory network (Wellmer and Riechmann 2010). To verify the most likely candidate gene, the expression of *LFY* and *AP1* homologous genes in the SAM were detected in RcBr and CSSL16. *LFY* homologous genes included *BraA02g043220.3C* and *BraA06g025360.3C*, and the *AP1* homologous gene included *BraA02g018970.3C*, *BraA07g030470.3C*, and *BraA07g034100.3C* in *B. rapa*. Five specific primers, RT-22, RT-36, RT-97, RT-47, and RT-41 were used to analyze the expression levels of the *AP1* and *LFY* genes (Table S2). The results showed that the expression levels of all the *LFY* and *AP1* homologous genes were significantly different between RcBr and CSSL16, with all the genes being downregulated in CSSL16 (Fig. 7). Higher expression of *LFY* and *AP1* result in *Arabidopsis* premature flowering, therefore, *ATC* (*BraA07g018240.3C*) possibly downregulates the expression of *LFY* and *AP1*, which would lead to delayed flowering, similar to the function of *ATC* in *A. thaliana*. In conclusion, we predicted that *BraA07g018240.3C* was the most likely candidate gene. To further confirm the candidate gene, we analyzed the sequence variations of *BraA07g018240.3C* between the two parental lines.

Sequence variation analysis of *BraA07g018240.3C*

To identify variations in the candidate gene sequence, a specific primer, 24-C, was used to detect CDS sequence variation (Table S2). The full length gene for *BraA07g018240.3C* is 1600 bp, starting at 15,558,430 and ending at 15,560,029, including three introns and four exons. The CDS sequence had an A to T mutation at the 12^{th} base in first exon and a base T to C change at the 32^{nd} base in the second exon, however, mutations were both synonymous (Fig. 4d). Two specific primers, QG-1 and QG-22 (Table S2), were used to amplify the promoter. The result showed many changes in the promoter regions of *BraA07g018240.3C*between two parental lines (Fig. S1).

Discussion

In this study, we employed BSR-seq based on secondary F_2 populations derived from CSSL16 and RcBr to identify the QTL *qFT7.1* (Fig. 3), which is responsible for late flowering in *B. rapa*, and was further validated using classical QTL mapping (Fig. 4c). QTL-seq (BSR-seq) combined with classical QTL mapping has proven to be a powerful tool to identify major QTLs controlling traits of interest in a variety of crops (Lu et al. 2014; Berenguer et al. 2015; Gelli et al. 2017; Shu et al. 2018; Park et al. 2019; Wen et al. 2019; Huang et al. 2021). Thus, most of the populations utilized for QTL-seq are preliminary populations, such as F_2, DHs, and RILs. QTL-seq utilized mainly for detecting major QTLs with large effects, QTLs with minor effects might not be detected by QTL-Seq, as traditional QTL mapping did. Recently, Zhang et al. (2019) devised a new method, quantitative trait gene sequencing (QTGseq), to accelerate QTL fine mapping through QTL partitioning and wholegenome sequencing of BSA populations. QTL partitioning is a strategy used to convert a quantitative trait into a near-qualitative trait quickly in nature. However, compared with the line developed by QTG-seq, CSSLs (or NILs) showed a relatively homozygous genetic background, only the target region is a substitution fragment, and other regions are not replaced; therefore, the background is basically the same as the recurrent parent, and the positioning accuracy was

relatively high. Furthermore, transcriptional analysis (RNA-seq) based on CSSLs (or NILs) also has advantages for identifying candidate genes underlying QTLs (Keurentjes et al. 2007).

Ultimately, *qFT7.1* was fine mapped to a 56.4-kb interval, between the two InDel markers, InDel714 and InDel715, (Fig. 4c) and a physical position of 15,539,588 to 15,595,959 on chromosome A07. In our previous studies, we did not detected any QTLs in the candidate region basing on F_2, RIL, and CSSLs derived from the identical parent, RcBr and 08A061 (Wang et al. 2014; Liu et al. 2016; Wang et al. 2018b). The CSSLs were constructed using 166 InDel and SSR markers that were distributed relatively evenly on the ten chromosomes; however, a low marker density is likely to lead to small introgression segments being missed. We did not detect any introgression segments on chromosome A07 for CSSL16 based on a limited number of markers (data not shown); therefore, we re-sequenced the two parental lines to identify the possible segment derived from 08A061 (Fig. 1). Until now, no other flowering related QTLs were detected in the candidate interval of *qFT7.1* on chromosome A07 in *B. rapa*, allowing us to identify the *Arabidopsis ATC* homologous gene for the first time, which is of a great significance to breed late flowering varieties of *B. rapa.*

ATC is a *TFL1*-like gene in *Arabidopsis* that is homologous with *CENTRORADIALIS* (*CEN*) and was first identified in *Antirrhinum* (Bradley et al. 1996). *ATC* inhibits flowering and regulates inflorescence morphology (Banfield and Brady 2000). *ATC* encodes a protein that is 77% similar to *Antirrhinum* CEN and 67% similar to *TFL1* at the amino acid level. *ATC*, *CEN*, and *TFL1* overexpression showed similar phenotypes in wild-type *Arabidopsis* (Ratcliffe et al. 1998; Mimida et al. 2001). *ATC* acts in a non-cell autonomous manner to inhibits flowering in *Arabidopsis*, and is specifically expressed in the hypocotyl, through long distance movement to the SAM. *LFY* and *AP1* are the critical factors that regulate FT positively in the SAM. *ATC* might inhibit the expression of *LFY* and *AP1* to delay flowering (Hempel et al. 2000; Huang et al. 2012; Gao et al. 2017). Many *ATC* homologous genes have been identified in different species, such as *GoCEN-Dt* in cotton, *Hordeum vulgare CENTRORADIALIS* (*HvCEN*) in barley, *SELF-PRUNING* (*SP*) in tomato, and *ZEA CENTRORADIALIS* (*ZCN*) in maize (Pnueli et al. 1998; Danilevskaya et al. 2010; Coradran et al. 2012; Liu et al. 2018). These studies indicated that *ATC* homologous proteins have similar functions and their upregulated expression delays flowering. Our study also found that the *BraA07g018240.3C* expression level in CSSL16 was higher than that in RcBr, which fits with the prediction that upregulated expression of this ATC-like gene would delay the flowering; however, this function needs to be verified in transgenic plants.

BraA07g018240.3C was homologous with *Arabidopsis* gene *ATC* (*At2g27550*), which belongs to the *FT* family and acts systemically to inhibit floral initiation. We found that *BraA07g018240.3C* was expressed specifically in the hypocotyl and root, which was consistent with the results of Huang et al. (2012), in *Arabidopsis*, the *ATC* gene is mainly expressed in vascular tissues, but not in the apex. In the present study, we could not detect any expression (the number of reads was zero) of *BraA07g018240.3C* based on BSR-seq, which proved that *BraA07g018240.3C* is a specially expressed gene in *B. rapa*. According to our results, the expression of *AP1* and *LFY* genes in the SAM was significantly higher in RcBr than in CSSL16, which, consisted with the results reported by Liu et al (2009) and Kaneko-Suzuki et al. (2018),

allowed us to speculate that *ATC* might inhibit *AP1* and *LFY* expression positively by long distance transport in the SAM, which then delays flowering in *B. rapa*.

We cloned the candidate gene *BraA07g018240.3C* promoter and CDS sequence, and identified two synonymous mutations in the CDS and some mutated bases in the promoter sequence. Changes in the promoter region might influence gene expression, for example, tomato *SELF PRUNING 5G* is a critical gene for FT, and Zhang et al (2018b) found that changes to the promoter region resulted in delayed flowering. The candidate gene for a major QTL controlling tomato weight, *fw2.2*, also had changes in its promoter sequence, which influenced fruit weight (Nesbitt and Tanksley. 2002). In *B. rapa*, Su et al (2021) identified sequence variations in the promoter of *BrHISN2*, which conferred cold-dependent expression on *BrHISN2*, resulting in leaf yellowing. Thus, the significantly different expression levels of *BraA07g018240.3C* between the two parental lines might be caused by changes in the promoter region.

In conclusion, we fine mapped a novel QTL for FT, *qFT7.1*, to a physical interval of 56.4 kb on chromosome A07. The CSSL16 allele at the *qFT7.1* locus regulates the FT negatively at the bolting stage of *B. rapa*. According to the sequence and expression level, the most likely candidate gene, *BraA07g018240.3C*, encodes a *TFL1*-like protein. These findings could aid our understanding of the mechanisms underlying flower formation and provide a genetic resource for *B. rapa* crop improvement studies.

Declarations

Author contribution statement

YW designed the experiments. YG, XW, YS, XG, WW and CH helped developing the CSSLs. GQ conducted the experiments, wrote the manuscript and performed the data analysis, HF and WF assisted in the data analysis. GQ and YW revised the manuscript. All authors reviewed and approved this submission. The authors note that this research was performed and reported in accordance with ethical standards of the scientific conduct.

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Code availability Not applicable, software used according to manuals.

Ethical statement

The authors note that this research was performed and reported in accordance with ethical standards of the scientific conduct.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Availability of data and material

The datasets generated and analyzed during this study are available on reasonable requests from the corresponding authors.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Tables

Table 1. Phenotypic values for RcBr[1] and CSSL16 under four environments

Trait	DE5 (days)		DE10 (days)		FT (days)		BI	
Environment	RcBr	CSSL16	RcBr	CSSL16	RcBr	CSSL16	RcBr	CSSL16
E1	40.21 ± 0.94	52.36 ± 1.35**	49.33 ± 2.27	61.58 ± 2.18**	75.47 ± 3.78	98.19 ± 2.07**	7.00 ± 0.00	3.00 ± 0.00**
E2	35.00 ± 1.36	41.71 ± 1.20**	40.46 ± 1.24	44.00 ± 0.88**	52.26 ± 1.27	63.43 ± 1.28**	7.00 ± 0.00	5.00 ± 0.00**
E3	21.00 ± 1.91	27.71 ± 0.09**	25.64 ± 1.65	33.23 ± 0.44**	44.21 ± 3.55	61.69 ± 3.88**	7.00 ± 0.00	5.00 ± 0.00**
E4	19.23 ± 1.12	24.32 ± 0.65**	23.24 ± 1.73	29.96 ± 2.04**	38.78 ± 2.78	51.55 ± 3.37**	7.00 ± 0.00	5.00 ± 0.00**

E1–E4 are four different environment as same as Table S1. Data are presented as means ± standard errors. significant levels:*P < 0.05,**P < 0.01’

[1]Abbreviations: RcBr, rapid-cycling inbred line of *B. rapa*; CSSL16, chromosome segment substitution line 16; DE5/DE10, days to reach a 5/10-cm-high elongated floral stalk; FT, flowering time; BI, bolting index.

Figures

Figure 1

Sequence variations between RcBr and CSSL16 detected using whole-genome resequencing. Distribution of SNP-EDs and INDEL-EDs screened on chromosomes based on whole-genome resequencing. The x-axis

represents the 10 B. rapa chromosomes and the y-axis shows the ED values of the filtered SNP-EDs and INDEL-EDs. a The distribution of SNP-EDs. b The distribution of INDEL-EDs

Figure 2

Phenotypic characterization of RcBr and CSSL16. a The early flowering line ‘RcBr’ (left) and late flowering line ‘CSSL16’ (right) grown under natural conditions. b Column diagram evaluating the flowering time of the two lines in four different environments (E1, E2, E3 and E4). Data presented are means with SD (n=30 plants). Scale bars = 5 cm, **Significant at P < 0.01

Figure 3

The distribution of the ED5 value of differential SNPs on Brassica rapa chromosomes according to BSR-Seq analysis. BSR-Seq-based distribution of SNPs on chromosomes. The x-axis shows the 10 B. rapa chromosomes and the y-axis shows the ED5 values of the filtered SNPs, the horizontal line is the threshold of the top 1%

Figure 4

Fine mapping of qFT7.1 a Graphic representation of the genotype of chromosome A07 encompassing qFT7.1. Sequence variations of chromosome A07 between the two parental lines detected by whole genome re-sequencing. The orange region represents the confidence interval of qFT7.1 identified by BSR-seq. b Traditional QTL mapping was preformed to validate the QTL qFT7.1. Physical map of the qFT7.1 region on chromosome A07. Traditional QTL analysis was used to detect qFT7.1, which was preliminary located between marker InDel714 and InDel716. The position of markers is shown on the x-axis and the LOD value is shown on the y-axis. The LOD value of qFT7.1 was 32.2, which explained the rate 39.9% of

the phenotypic variation. c Fine mapping of qFT7.1. The phenotype and genotype of the ten homozygous recombinant groups and the two parental lines (RcBr and CSSL16) used for fine mapping. The marker genotypes of RcBr are shown as black bars and those of CSSL16 are shown as white bars, DE5, DE10, and FT data appear as means ± SD. Significant differences for the traits of the recombinant compared with those of the parents are indicated using superscript letters (a, b). Student's t-test was used to distinguish significant difference at $P < 0.01$. d Structure of the BraA07g018240.3C coding region. Whole genome re-sequencing and cloning detected sequence variation of BraA07g018240.3C between the two parental lines, the black regions represent exons and the straight lines represent intron, two base variation mutations were identified in first and second exons

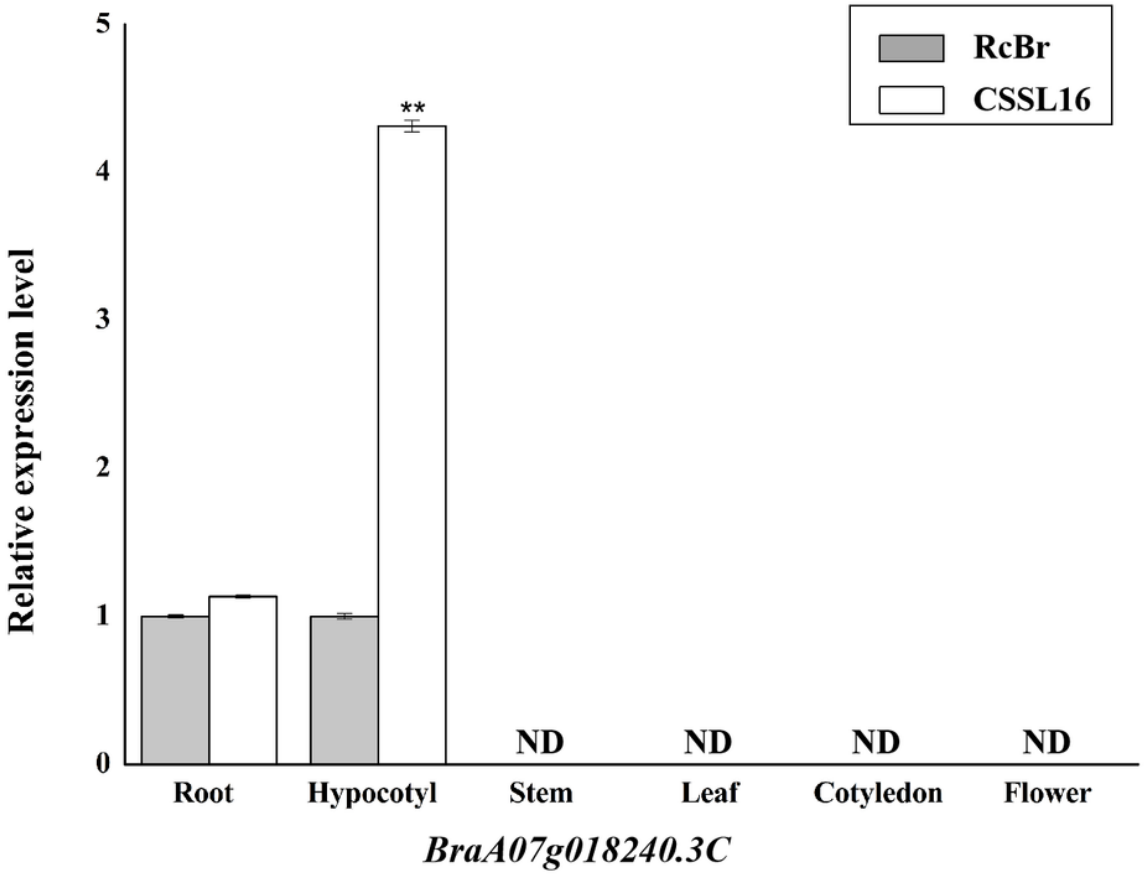

Figure 5

Expression patterns of BraA07g018240.3C in plant tissue BraA07g018240.3C expression in the root, hypocotyl, stem, leaf, cotyledon and flower, assessed using qRT-PCR. BraA07g018240.3C was especially expressed in the root and hypocotyl, and showed a significant difference in the hypocotyl. Error bars represent the standard errors from three replications, $P < 0.01$

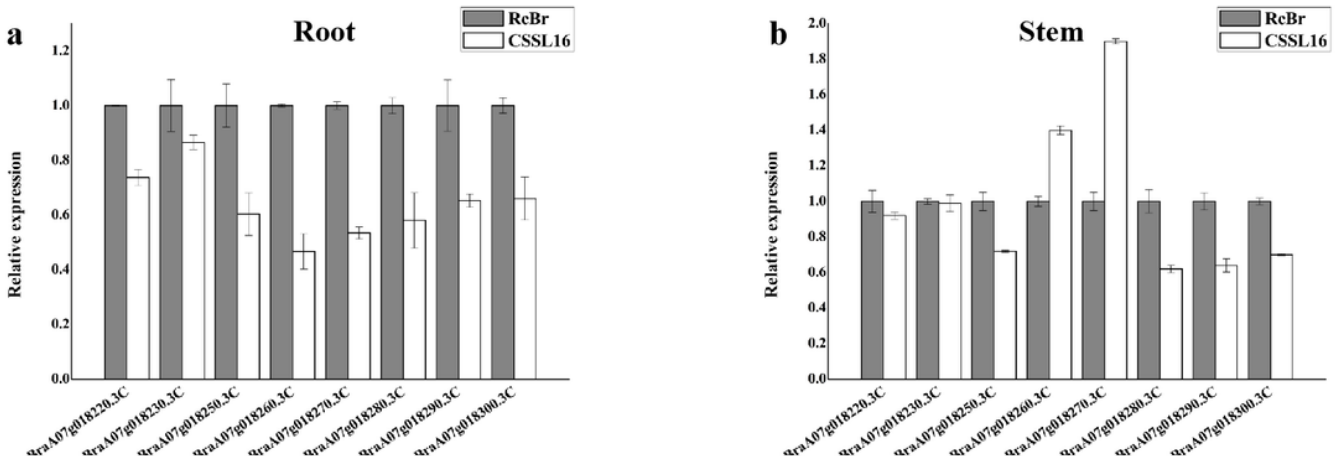

Figure 6

Expression level of eight genes in the candidate region Expression level of eight genes in (a) the root, (b) stem, (c) leaf and (d) flower determined using qRT-PCR. The expression levels of BraA07g018270.3C and BraA07g018300.3C showed significant differences in flower. Error bars represent the standard errors from three replications, $P < 0.01$

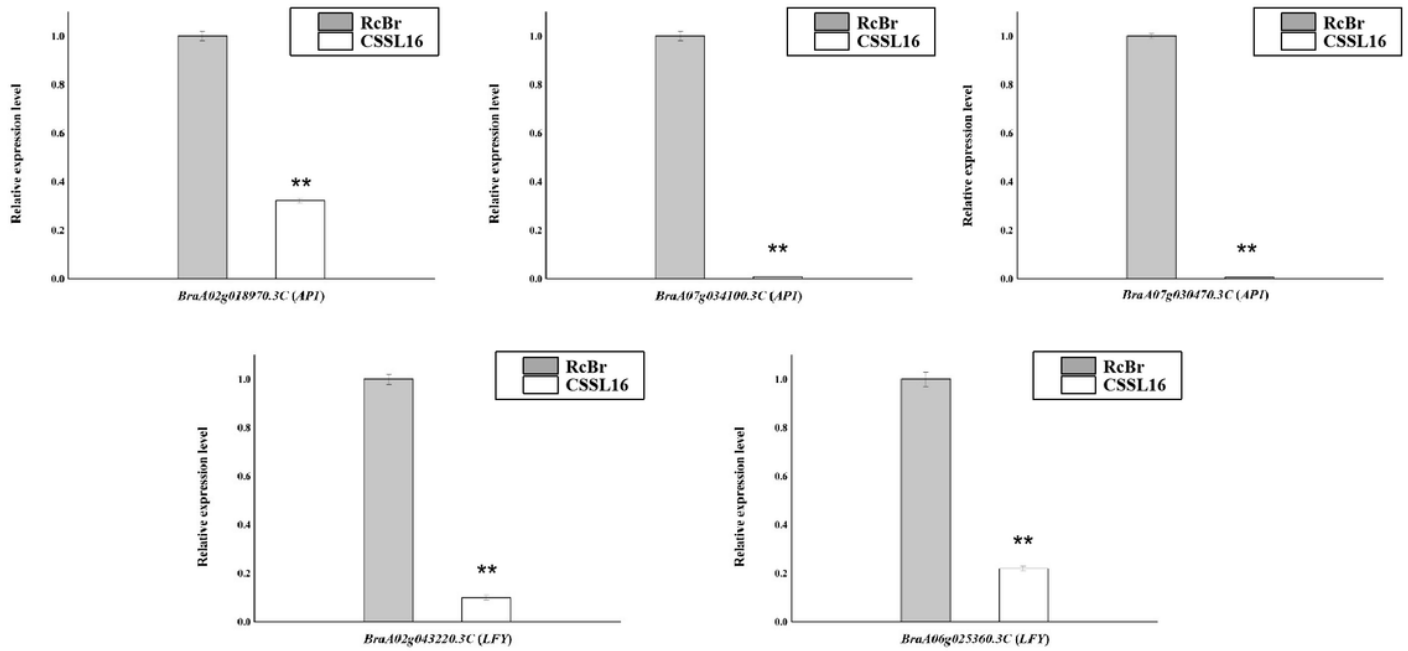

Figure 7

Expression level of an ATC-related genes in the flowering stage Expression of AP1 and LFY genes in B. rapa according to flowering time. The expression levels of AP1 (BraA02g018970.3C, BraA07g034100.3C, BraA07g030470.3C) and LFY genes (BraA02g043200.3C, BraA06g025360.3C) in 'RcBr' and 'CSSL16'. Error bars represent the standard errors from three replications, $P < 0.01$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.tif
- TableS1.doc
- TableS2.xlsx
- TableS3.xls