Photoperiod responsiveness is important to soybean production potential and adaptation to local environments. Varieties from temperate regions generally mature early and exhibit extremely low yield when grown under inductive short-day (SD) conditions. The long-juvenile (LJ) trait is essentially a reduction and has been introduced into soybean cultivars to improve yield in tropical environments. In this study, we used next-generation sequencing (NGS)-based bulked segregant analysis (BSA) to simultaneously map qualitative genes controlling the LJ trait in soybean. We identified two genomic regions on scaffold_32 and chromosome 18 harboring loci LJ32 and LJ18, respectively. Further, we identified LJ32 on the 228.7-kb scaffold_32 as the soybean pseudo-response-regulator gene Tof11 and LJ18 on a 301-kb region of chromosome 18 as a novel PROTEIN FLOWERING LOCUS T-RELATED gene, Glyma.18G298800. Natural variants of both genes contribute to LJ trait regulation in tropical regions. The molecular identification and functional characterization of Tof11 and LJ18 will enhance understanding of the molecular mechanisms underlying the LJ trait and provide useful genetic resources for soybean molecular breeding in tropical regions.

KEYWORDS
long-juvenile, BSA, positional cloning, Tof11, FT
Introduction

Soybean [Glycine max (L.) Merr.], the main source of vegetable protein and oil globally, is a facultative short-day crop (GrahamVance, 2003). Flowering time and maturity traits significantly determine both plant adaptation to specific latitude and grain yield (Cober and Morrison, 2010; Zhong and Kong, 2022). Soybean is cultivated in a widespread latitude range, from high-latitude areas such as Northeast China to tropical regions such as South America (Wang et al., 2016; Han et al., 2016). This broad ecological adaptability is enabled by genetic variation at major gene loci and quantitative trait loci (QTLs) controlling flowering and maturity (Lin et al., 2021a; Lin et al., 2021b). Multiple naturally occurring variants at these loci have become the targets of human selection and endow soybean with the flexibility to adapt to different areas with distinct photoperiod patterns. To date, 16 maturity loci, E1 to E11, J, Tof5, Tof11, Tof12, and LUX, have been identified by forward-genetic approaches (Bernard, 1971; Buzzell, 1971; McBlain and Bernard, 1987; Ray et al., 1995; Bonato and Vello, 1999; Cober and Voldeng, 2001a; Cober et al., 2010; Xia et al., 2012; Kong et al., 2014; Li et al., 2017; Samanfar et al., 2017; Wang et al., 2019; Lu et al., 2020; Bu et al., 2021; Dong et al., 2022). Among them, E1, E2, E3, E4, E7, E8, E10, Tof11, and Tof12 delay flowering and maturity under long-day (LD) conditions, and their recessive alleles enhance soybean adaptation to high latitudes (Cober and Voldeng, 2001b; Liu et al., 2008; Watanabe et al., 2009; Cober et al., 2010; Xia et al., 2012; Cao et al., 2017; Samanfar et al., 2017; Wang et al., 2019; Lu et al., 2020). A recent report indicated that the J protein associates with two LUX homologs to form the evening complex, which plays key roles in photoperiodic flowering and photoperiod sensitivity in soybean under both short-day (SD) and LD conditions (Bu et al., 2021). Tof11 and Tof12, encoding two homologous pseudo-response regulator (PRR) proteins, improved adaptation to the limited summer growth period at higher latitudes during soybean domestication (Lu et al., 2020).

At the other end of the latitudinal range, in the tropics, warm temperature and short photoperiod strongly induce rapid flowering and early maturity in photoperiod-sensitive soybean cultivars, making the vegetative phase very short and resulting in low yields (Parvez and Gardner, 1987; Destro et al., 2001). In these conditions, extension of the reproductive and phasic phases is necessary to allow greater vegetative growth and improve yield. The long-juvenile (LJ) trait has been introduced into tropical soybean cultivars to meet this need (Sinclair and Hinson, 1992; Carpentieri-Pipolo et al., 2002; Li et al., 2017; Lu et al., 2017). However, genetic information regarding this trait remains limited. As the major classical locus conferring the LJ trait, J was identified as the ortholog of Arabidopsis thaliana EARLY FLOWERING 3 (ELF3). J depends genetically on the legume-specific flowering repressor E1 and directly downregulates E1 expression, thereby relieving the repression of two important FLOWERING LOCUS T (FT) genes (FT2a and FT5a) and promoting flowering under SD conditions (Lu et al., 2017; Fang et al., 2021). Recently, FT2a and FT5a were found to have variants of diverse origins that played distinctive roles as soybean spread to lower latitudes (Li et al., 2021). Tof16 was identified as a novel LJ locus that harbors the soybean homolog of the Arabidopsis LATE ELONGATED HYPOCOTYL (LHY), which delays flowering and improves yield at low latitudes (Dong et al., 2021). Loss of function of J, FT2a, or Tof16 is the major genetic base of soybean adaptation in tropical regions. Additionally, many QTLs associated with the LJ trait have been identified in soybean varieties (Fang et al., 2019; Lin et al., 2021a).

Conventional positional cloning and QTL mapping are powerful approaches for investigating the genetic control of phenotypic variation in agronomic traits (Burke et al., 2007). However, classical map-based gene cloning approaches are usually time-consuming owing to the need for genetic crossing and phenotypic analysis. As an alternative, the application of bulked segregant analysis (BSA) to QTL selection provides a simple strategy for rapidly identifying molecular markers tightly linked to the causal gene underlying a given phenotype (Giovannoni et al., 1991; Michelmore et al., 1991). BSA methods have been used in many organisms to map important genes (Mansur et al., 1993; Yi et al., 2006; Watanabe et al., 2011; Whipple et al., 2011). With the continuing advances in DNA sequencing technology, next-generation sequencing (NGS)-based BSA can dramatically accelerate the process of identifying causal genes of particular traits (Schneeberger and Weigel, 2011).

To investigate QTLs and corresponding candidate genes associated with the LJ trait in this study, we used genome-wide NGS-based BSA mapping of a soybean biparental population to identify two QTLs, named LJ32 and LJ18, conferring the LJ trait in soybean. We further validated these two QTLs and fine-mapped them by marker-based classical gene mapping to two intervals of 229 and 301 kb. Molecular and transgenic analyses demonstrated that the PRR gene Time of Flowering 11 (Tof11) and a PROTEIN FLOWERING LOCUS T-RELATED gene, Glyma.18G298800, may be responsible for the effects of the LJ32 and LJ18 loci. Overall, our study provides a useful genetic resource for soybean adaptation and molecular breeding to adapt soybeans for growth in tropical environments.

Results

Phenotypic analysis

To identify additional loci contributing to the LJ trait, we developed a set of 213 recombinant inbred line (RIL) populations from a cross between two closely related soybean cultivars. We used the near-isogenic lines (NILs) ZK193 and ZK158, both with the genetic background of the Canadian cultivar Harosoy from L62-812, which have the same genotypes (e1/e2/E3/E4/E9/Dt1) for the major flowering time...
genes E1-E4 and E9 and the stem growth habit gene Dt1 (Supplementary Table 1). Nonetheless, ZK193 shows significantly earlier flowering and maturity than ZK158 under SD conditions (12 h light/12 h dark) (Figure 1). In addition, the two parents displayed different phenotypes in regard to several other traits, including node and pod number, grain number, and grain yield per plant (Supplementary Figure 1). To perform QTL mapping, we created cross combinations by pollinating ZK193 with pollen from ZK158 to develop descendant populations. We planted all RIL individuals and plants of both parent strains across 2 years (2018 and 2019) in Guangzhou, China, and recorded their flowering times at the R1 stage. The flowering data from 2018 and 2019 were strongly correlated (P < 0.01, R = 0.724), and we therefore used the data from 2019 for the following bulk segregation analysis (BSA) to detect flowering-associated loci.

Analysis of flowering-associated loci by BSA and QTL mapping

Based on the phenotypic assessment, we pooled genomic DNA from 30 individuals with extreme phenotypes (extremely early flowering and extremely late flowering) separately into an EF bulk sample and a LF bulk sample, respectively. We also extracted genome DNA from each parental line, isolated from leaves of 20 plants, for NGS-based BSA sequencing. After filtering, we identified clean reads and aligned them to the Williams 82 reference genome, and obtained high-quality single-nucleotide polymorphisms (SNPs) with which to calculate SNP-index values. We observed two peaks in the SNP-index plot, which we assigned as candidate flowering-time control regions in this population (Figure 2). The candidate regions were designated LJ trait 32 (LJ32) and LJ18 due to their locations on scaffold_32 and chromosome 18, respectively. SNP-index analysis revealed that the regions of the physical map around 229 kb on scaffold_32 and 4.7 Mb on chromosome 18 might be associated with flowering time (Supplementary Table 2, Supplementary Figure 2).

Scaffold_32 is a fragment that failed to be successfully assembled in the Williams 82 reference genome. Because it is a small fragment (229 kb), we performed ANOVA analysis to confirm the flowering-associated interval on Scaffold_32 (Supplementary Table 2). The results showed that the flowering times of all plants with the ZK193 marker pattern for Scaffold_32 were significantly earlier than the mean value for plants with the ZK158 pattern. We inferred that a QTL is present in the same chromosome region as the marker Tof11, here named LJ32. Under SD conditions, LJ32 significantly affected flowering time, as shown by genome-wide analyses with permutation tests (P < 0.05) (Figure 2). To validate the candidate region chromosome 18 identified by BSA mapping, we used numerous insertion-deletion (Indel) and SNP markers in the region that are polymorphic between the two parental lines to genotype and constructed genetic linkage maps in the RIL population using the Kosambi function. A total of 11 markers, spanning 67.8 cM (Supplementary Figure 2), covered a part of the region of linkage group 18. The main marker type contributing to this linkage map was Indel markers, while the linkage gaps between the Indel markers were bridged by SNP markers. The constructed map was generally consistent with the US Department of Agriculture soybean genetic linkage map (Choi et al., 2007). QTL analyses revealed that LJ18 was located in a region between markers ID181191 and ID181120 on chromosome 18 (Supplementary Table 2). Two significant QTLs for flowering time, LJ32 and LJ18, were consistently detected in the 2018 and 2019 data, validating the accuracy of the NGS mapping results.

Characterization of LJ32

On the Scaffold_32 fragment, 22 genes were annotated. Of these, Glyma.U034500, encoding a PRR family protein, was
identified as Tof11, which has been reported to delay flowering in LD conditions (16 h light/8 h dark) (Lu et al., 2020) (Supplementary Table 3). Upon further analysis of the NGS data, we identified a 1-bp deletion (A2210-) in the last exon of Tof11, causing frameshifts and premature termination of protein translation, in the parent ZK193 compared with that in ZK158 (Supplementary Figure 3). Previous study found that this Tof11 haplotype, which we named Tof11-1, was the most abundant in landraces and improved cultivars and identified to be selected at an early stage of modern soybean breeding, and Tof11 is genetically dependent on E1 (Lu et al., 2020). We evaluated the effect of Tof11 on transcriptional regulation of E1 under SD conditions. A similar result was obtained in the parents showing that functional alleles of Tof11 in ZK158, relative to the respective mutant alleles in ZK193, increased E1 expression (Supplementary Figure 4). We thus identified Tof11 as a candidate gene potentially responsible for the effect of LJ32.

To characterize the function of Tof11 in soybean LJ regulation, we grew two complementary transgenic Tof11 lines (TC#2 and TC#4), along with a wild-type (WT) cultivar Dongnong 50 (Lu et al., 2020), under SD conditions. The TC#2 and TC#4 plants flowered slightly but significantly later than WT plants under SD conditions (Figure 3), supporting our hypothesis that LJ32 is encoded by TOF11.

To further validate the function of Tof11 in soybean LJ trait regulation under SD and identify the allelic variations of Tof11, we looked for variations in the Tof11 coding sequence in our collection of 338 re-sequenced soybean accessions from low-latitude regions grown in Guangzhou (Li et al., 2021). We identified 11 haplotypes, of which H2 and H4 (functional alleles) resulted in significantly later flowering than H1 and H3 (loss-of-function alleles) (Figure 4). The remaining alleles were not assessed because they were found in only a few accessions (Figure 4). Notably, in these 338 accessions, the frequency of functional alleles (48%) was similar to that of loss-of-function alleles, indicating that variations in Tof11 may contribute to the geographic distribution of soybean accessions in lower-latitude regions. Together, our observations indicated that Tof11 is the most likely causal gene in the LJ32 locus for the LJ trait.
Positional cloning of LJ18

To further delineate the LJ18 locus, we surveyed the genotypes at two markers within the QTIL in 1354 plants segregated from heterozygous plants and detected five recombinants. We also investigated the segregation pattern in the residual heterozygous lines (RHLs) (Supplementary Figure 5). Fine-mapping with seven additional molecular markers delimited the LJ18 genomic region to an ~301-kb region between markers M4 and M5 (Figure 5), which harbors 25 genes according to the Williams 82 reference genome (Supplementary Table 4). Among them, three PROTEIN FLOWERING LOCUS T-RELATED (GmFT1) genes were annotated: Glyma.18G298800, Glyma.18G298900 (GmFT1a), and Glyma.18G299000 (GmFT1b) (Supplementary Figure 6). We found no variants in GmFT1a resulting in amino acid changes and one non-synonymous SNP each in GmFT1b and Glyma.18G298800 (LJ18) (Supplementary Figure 6, 7A).

GmFT1a and GmFT1b are widely recognized as FLOWERING LOCUS T (FT) homologs in soybean (Kong et al., 2014), and variants in promoter regions can regulate gene expression and function (Liu et al., 2018; Li et al., 2021). We therefore tested the functions of each gene in soybean flowering regulation using loss-of-function mutants generated by CRISPR/Cas9 gene editing in the Williams 82 cultivar. We obtained multiple mutants for both GmFT1a and GmFT1b (Supplemental Figures 7B, C). Unexpectedly, phenotypic analysis detected no significant difference in flowering time between the Williams 82 and these single mutants under SD conditions (Supplementary Figure 7D).

Another FT-related protein, encoded by Glyma.18G298800, was annotated and located in tandem with GmFT1a and GmFT1b on chromosome 18 (Supplementary Table 4). NGS data revealed a non-synonymous SNP in the 3rd exon specifying a polymorphism between the two parental strains at residue T170C (T57M), which is threonine (T) in ZK158 but methionine (M) acid in ZK193 (Figure 5C). These observations suggested that Glyma.18G298800 is a probable candidate for the causative gene in the LJ18 locus. We investigated the allelic variation of Glyma.18G298800 using the same strategy described above for ToF11 and identified five haplotypes, among which haplotype 2 (H2) corresponded to the lj18 allele (Figure 6A).
the geographic distribution of various alleles at LJ18 loci within the 338 accessions including the five LJ18 alleles (Figure 6A). All alleles showed no significant geographical distribution characteristics (Supplementary Table 5). We also analyzed the association of the Glyma.18G298800 haplotypes with flowering time in Guangzhou over 2 years. Accessions carrying H2 and H3 flowered significantly later than those carrying H1 (Figure 6B, C). This observation indicated that the polymorphism at nucleotide 170 in Glyma.18G298800 may lead to the variation in flowering time, supporting a role for Glyma.18G298800 in the control of flowering time in diverse genetic backgrounds. Taking these results together, we suggest that Glyma.18G298800 is a
likely candidate gene for LJ18 and influences flowering regulation under SD conditions.

Functional analysis of Glyma.18G298800 in soybean flowering regulation

To confirm the expression patterns of Glyma.18G298800, we used reverse transcription quantitative PCR (RT-qPCR) to analyze the expression patterns of Glyma.18G298800 in leaves at different development stage of Williams 82 soybean (Supplemental Figure 8A). We found that Glyma.18G298800 transcripts were much more abundant in cotyledons and leaves than in other tissues. To preliminarily examine the function of Glyma.18G298800, we ectopically expressed Glyma.18G298800 in the Arabidopsis Columbia-0 (Col-0) ecotype. Among the resulting transgenic lines, RT-qPCR results showed that two independent lines exhibited relatively higher Glyma.18G298800 transcript levels than Col-0, and we selected these for further phenotypic analysis (Supplemental Figure 8B). The results showed that overexpression of Glyma.18G298800 significantly promoted flowering time compared with that of Col-0 (Figures 7A–C).

Next, we explored the molecular mechanism underlying the effect of Glyma.18G298800 on the LJ trait through a dual-luciferase assay. As soybean APETALA1 (AP1) is reported to be the primary target for FT regulation (Chen et al., 2020; Li et al., 2021), we used this assay to examine the effect of Glyma.18G298800 on AP1a, by fusing a 3-kb fragment of the AP1a promoter to the luciferase (LUC) reporter gene. We used Glyma.18G298800H1 and Glyma.18G298800H2 driven by the CaMV 35S promoter as effectors. We transformed each effector construct, together with the reporter construct, into N. benthamiana. Compared with the reporter vector control, the Glyma.18G298800H1 effector promoted the activity of AP1a promoter, as revealed by an increased LUC/REN (Figure 7D, E). The Glyma.18G298800H2 effector resulted in a lower LUC/REN ratio than Glyma.18G298800H1 effector, indicating that Glyma.18G298800H2 has no effect on the activity of the AP1a promoter (Figure 7D, E), which is consistent with the parental flowering phenotypes and with the results of the haplotype analysis above. Collectively, our results demonstrated that the PROTEIN

![Figure 7](image_url)
FLOWERING LOCUS T-RELATED gene Glyma.18G298800 may function as a flowering promoter in soybean.

Discussion

In tropical regions, days are short during the growing season, neither temperature nor water are limiting, and the long-juvenile (LJ) trait is well established as an important adaptation that allows the crop to take full advantage of this favorable environment (Li et al., 2021). The introduction of the LJ trait in the 1970s overcame limitations on soybean growth, allowing its production to be extended to lower-latitude (tropical) areas (Neumaier and James, 1993; Carpentieri-Polo et al., 2002). For example, until 1960, soybean cultivars used in Brazil were imported from the United States, and cultivation areas were limited to above 22 degrees south latitude. In recent decades, however, the introduction of LJ germplasm has enabled Brazil to become the world’s second-largest soybean producer (Neumaier and James, 1993). Notwithstanding the importance of LJ genes for soybean adaptation and yield in tropical regions, however, the underlying genetic basis and the trajectory of adaptation to low latitudes by means of these genes have remained largely unknown.

In this study, we developed a hybrid population from crosses between the NILs ZK193 and ZK158. NGS-based BSA combined with QTL analysis revealed two QTLs associated with the LJ trait, Lj32 and Lj18, located on scaffold_32 and chromosome 18, respectively, in the Williams 82 reference genome. Scaffold_32 is a 229-Kb fragment that is assembled on chromosome 11 of the Zhonghuang 13 reference genome (Lu et al., 2020). We considered the gene Tof11, located in this region, as a candidate for the Lj32 locus. Although Tof11 is reported to delay flowering under LD conditions and to have contributed to ancient flowering-time adaptation (Lu et al., 2020), its function in LJ regulation had not previously been investigated. Here, we demonstrated that Tof11 functions as a flowering inhibitor under LD conditions and regulates the soybean LJ trait at low latitude.

FT proteins comprise a clade of the plant phosphatidylethanolamine-binding protein (PEBP) family and act as highly conserved regulators that are pivotal in the flowering pathways of various crop species (Kong et al., 2010). In soybean, several FT homologs have been reported as candidate florigens or antiflorigens under LD or SD conditions, including GmFT2a, GmFT5a, GmFT1a, and GmFT4 (Zhao et al., 2016; Liu et al., 2018; Jiang et al., 2019; Lu et al., 2020). Furthermore, GmFT2a and GmFT5a act as floral promoters conferring the LJ trait and played distinct roles as soybean spread to lower latitudes (Nan et al., 2014; Cai et al., 2020; Li et al., 2021; Yue et al., 2021). Here, we report that Glyma.18G298800, a novel FT homolog, is a possible candidate for the Lj18 locus that regulates the soybean LJ trait. This finding provides preliminary evidence that Glyma.18G298800 may contribute to delaying the flowering time of soybean varieties by inhibiting AP1a expression.

In summary, we used NGS-based BSA combined with QTL analysis to reveal two different QTLs conferring the LJ trait. We identified LI32 as the soybean PRR gene Tof11 and Lj18 as the PROTEIN FLOWERING LOCUS T-RELATED gene Glyma.18G298800. The natural variants of both genes have significant influence on flowering time in SD accessions, suggesting that these two genes may play important roles in controlling flowering time in tropical regions. The identification and characterization of these LJ-related genes will contribute to the understanding of the genetic and molecular mechanisms underlying the LJ trait and could be used to ensure the successful deployment of high-yield germplasm in tropical environments.

Materials and methods

Plant materials, growth conditions, and phenotyping

The NILs lines, ZK193 and ZK158 with genetic background of Canadian cultivar Harosoy from L62-812 were used; they have the same maturity genotypes at E1, E2, E3, and E4 (e1/e1 e2/e2 E3/E3 E4/E4 DT1/DTD1) (Supplementary Table S1). The cross combinations were made by pollinating ZK193 with pollen from ZK158 to develop RIL populations that were used for (NGS)-based Bulked segregation analysis (BSA) (Supplementary Table S2). The populations and low-latitude-adapted accessions for phenotyping were grown under naturally SD conditions (12 h light/12 h dark) in the field from 2017 to 2018 at the experimental station of Guangzhou University, Guangzhou (22° 26′ N, 112° 57′ E). The transformants for phenotyping were sown in pots in growth cabinets under SD conditions (12 h light/12 h dark)

Days to flowering were recorded at the R1 stage (first open flower appeared) for each plant. The R1 values reported for the parents and populations are means from 10 plants. The number of parents and RIL plants used in each experiment are listed in Supplementary Table 2. Days to flowering (R1) were individually recorded and subjected to analysis of variance. Means of days to flowering among lines were compared with Tukey’s HSD test using the Statistica software 03J (StatSoft). Plant height, number of branches, number of nodes, number of pods per plant, number of grains per plant, and yield per plant were all recorded at the R8 stage. All data are given as means ± s.e.m. (n = 10 plants). Two-tailed, two-sample t-tests were used to generate the P values.

DNA bulks construction and illumina sequencing

BSA was used to group the RIL population and its parents, Two DNA bulks for sequencing were first made by selecting
extreme individuals from the 213 RIL population plants with the basic statistics of the phenotypic data. One pool for early flowering comprised 30 lines with early flowering time and the other pool for late flowering involved 21 lines. DNA was extracted individually from leaves of plants, using a genomic DNA purification kit (Thermo Fisher Scientific Inc., United States) according to the manufacturer's protocol. The GC content, repeated sequences, and genetic characteristics of the DNA pools were analyzed by Biomarker (Beijing, China). About 20 μg of DNA from the two bulks and two parental lines were used to construct paired-end sequencing libraries. The genomic DNA pools were digested using the XhoI and MseI restriction enzymes (NEB, Ipswich, MA, USA), followed by PCR amplification, fragment amplification, fragment selection, fragment extraction and amplification, and fragment sequencing using the Illumina HiSeqTM 2500 (Illumina, Inc; San Diego, CA, USA) at Biomarker Technologies. Real-time monitoring was performed for each cycle during sequencing and the ratio of high-quality reads with quality scores greater than Q30 (a quality score of 30, indicating a chance of 0.1% for an error and thus 99.9% confidence) in the raw reads GC content was calculated for quality control.

After removing adapter and low-quality reads, the clean reads were further rechecked for quality control using FASTQC1. High-quality sequences were aligned and to the Glycine max Wm82.a2.v1 reference genome from Phytozome2 (https://phytozome-next.jgi.doe.gov) using BWA with default parameters (Langmead and Salzberg, 2012).

**SNP-index association analysis**

GATK (Genome Analysis Toolkit) was used to call SNPs and small indels across parental lines and bulks (McKenna et al., 2010). The relative marker abundance in bulked DNA pool 1 (the early flowering pool) was calculated as the number of reads of the paternal allele divided by the total of reads which then gives proportion paternal alleles (or alternatively maternal alleles), whereas in pool 2 (the late flowering pool), Homozygous SNPs between parental lines and high-quality SNPs (minimum sequence read depth: 10 with SNP base quality ≥ 100 in pools) were selected for SNP-index analysis. A SNP-index was calculated at each SNP position for both pools using the base in parental lines as alternative base (Abe et al., 2012; Takagi et al., 2013b). Thus, the SNP-index was assigned as 0 or 1, when entire short sequence reads contained genomic fragments derived from parental lines, respectively. A $\Delta$(SNP-index) was calculated by subtraction of the early flowering index from the late flowering index (Fekihi et al., 2013; Takagi et al., 2013a). Thus, a high $\Delta$(SNP-index) value of a SNP locus is indicative of an allele that was both very frequent in the pool 1 and depleted in the pool 2.

**QTL identification and statistical analysis**

The polymorphisms between the parents introduced two kinds of markers, SNP and Indel. Markers were developed based on re-sequencing data from the parents, ZK193 and ZK158. The whole genome re-sequencing of ZK193, ZK158 and the Indel analysis using the software of SOAPindel was conducted by BGI-Shenzhen, China as described previously (Kong et al., 2014). The procedures for polymerase chain reaction and gel-electrophoresis were adopted as reported earlier (Li et al., 2017). Marker order and distance were determined by Map Manager Program QTXb20 using the Kosambi function and a criterion of 0.001 probability (df. = 1) and a genetic map was constructed (Lu et al., 2015). Mapchart 2.1 was used to draw the linkage groups (Voorrips, 2002). The Multiple QTL Model (MQM), implemented by MapQTL 5.0 was used for QTL detection (Van Ooijen, 2004). A LOD score of 2.5 was used as a minimum to declare the significance of a QTL in a particular genomic region. The tests of 1000 permutations at a 0.05 probability were conducted to identify the genome-wide LOD score (Churchill and Doerge, 1994).

**Resequencing and variation calling**

The resequencing data, VCF files and flowering time data from the 338-accession panel used in this study were obtained from Li et al. (Li et al., 2021). The VCF files were processed using the VCFtools software (v.0.1.16). Paired-end resequencing reads of the 338 accessions were mapped to the Glycine max Wm82.a2.v1 (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax) with BWA software (Version: 0.7.17-r1188, http://bio-bwa.sourceforge.net/) with the default parameters. The duplicates of sequencing read for each accession were filtered with the Picard package (Version: V1.109, http://broadinstitute.github.io/picard/), and uniquely mapping reads were retained in BAM format. Reads around indels from the BWA alignment were realigned with the IndelRealigner option in the Genome Analysis Toolkit (GATK, Version: V3.2-2, https://gatk.broadinstitute.org/hc/en-us) and indel calling were performed with GATK and SAMtools software (Version: 1.9, http://samtools.sourceforge.net/). SNPs with MAFs less than 1% were discarded, and indels with a maximum length of 20 bp were included. SNP annotation was carried out based on the Williams82 genome, with Annovar (https://annovar.openbioinformatics.org/en/latest/).

**Fine Mapping of LJ18 locus**

For genetic analysis, we surveyed the genotypes by two markers for the F4 populations and conducted a segregating-heterozygous inbred family (n = 1354) that was heterozygous at LJ18 locus. The segregation pattern was carefully observed in the residual heterozygous lines (RHLs), in which the segregation
occurred only at LJ18 locus, seven additional Indel and SNP markers between markers ID181191 and ID181120 were identified (Figure 5A; Supplementary Table S5). Three recombinants in the region between M1 and M7 were genotyped using four Indel markers and three SNP markers (bold) (Figure 5A), and the flowering time of their progenies were evaluated to delimit the genomic interval containing LJ18. The genotypes of the LJ18 allele were analyzed by tagging marker M4 or M6 (Supplementary Table S5). The LJ18 allele was genotyped by its functional markers.

**Plasmid construction and plant transformation**

The CDS of the LJ18 candidate gene Glyma.18G298800 were obtained from ZK193 and ZK158. The CDS fragments were amplified by overlapping PCR to obtain one fragment and then introduced into the PTF101-Genevector (containing the bar gene for glucosinate resistance) (Li et al., 2021). The construct (PTF101-35S:LJ18) was next introduced into the Agrobacterium tumefaciens strain EHA105, and 35S:LJ18 was transformed into the PTF101-Genevector (containing the bar gene for glucosinate resistance) (Li et al., 2021). The construct (PTF101-35S:LJ18) was next introduced into the Agrobacterium tumefaciens strain EHA105, and 35S:LJ18-3flag transgenic lines were obtained through A. tumefaciens mediated transformation using the floral dip method in Col-0 wild-type followed by screening with 1/500 10% (w/v) basta. The FT1b knockout construct was produced by CRISPR-Cas9 as described previously (Ma et al., 2015). Two 20-bp sequences in the exons of FT1a and FT1b were selected as target sites for Cas9 cleavage (Supplementary Figure S6). Primers used for plasmid construction are listed in Supplementary Table S6. The above-mentioned CRISPR-Cas9 plasmid was transformed into Williams82 plants, and the transgenic plants were selected by basta (Ingbio, Lot: CB26213210).

**Gene expression analysis**

Soybean seedlings grown under SD conditions were harvested from the leaf of V3-stages for total RNA extraction using E.Z.N.A. Total RNA Kit I (Omega) and reverse transcribed to cDNA using MMLV-Reverse Transcriptase (Promega). qPCR was performed using a LightCycler 480 thermal cycler system (Roche) with KAPA SYBR Fast qPCR Kit Master Mix (Kapa Bio). The light intensity of the tobacco plant was measured using the Dual-Luciferase Reporter Assay System (Promega) under the manufacturers’ instructions. The LUC/REN ratio was measured from three biological replicates.

**Transient expression assays**

To generate the AP1a pro-LUC reporter construct, ~3 kb AP1a promoter was cloned into the pGreenII0800-LUC vector (Li et al., 2021). The Renilla Luciferase (REN) gene under the control of 35S promoter in the pGreenII0800-LUC vector was used as the internal control. The coding regions of 35Spro: LJ18-H1 and 35Spro: LJ18-H2 were cloned into the pGreenII62-SK vector and used as effectors. All primers used for these constructs are listed in Supplementary Table S6. The A. tumefaciens mixtures were infiltrated into three leaves of tobacco plants as described previously (Yue et al., 2021). p35S-LJ18-H1-LUC and p35S-LJ18-H2-LUC represent the A. tumefaciens carrying the effector constructs and the control vector pGreenII 0800-LUC. The tobacco leaves were allowed to recover for 48 h. LUC/REN activity showing the results from three independent replications and the value of each replication were represented by a dot. The LUC and REN activities were measured using the Dual-Luciferase Reporter Assay System (Promega) under the manufacturers’ instructions. The LUC/REN ratio was measured from three biological replicates.

**Data availability**

For phenotypic evaluation, at least ten individual plants were analyzed per accession, and the exact number of individuals (n) are presented in all figure legends. The exact number of replicates is given in figure legends. Mean values for each measured parameter were compared using one-way analysis of variance from SPSS (version 20, IBM) or one-tailed, two-sample Student’s t-tests from Microsoft Excel, whenever appropriate; the statistical tests used for each experiment are given in the figure legends. Whole-genome sequencing data for ZK193, ZK158, and the two bulks are deposited at CNCB-NGDC and are publicly available as of the date of publication. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**Data availability statement**

The data presented in the study are deposited in the “Bioproject” repository accession number “PRJNA896173”.

**Author contributions**

XL, FK, and BL designed the experiments, supervised the study, and managed the projects. TL, LW, CZ, and ShL performed most of the research. JW, SL, CF, LK, YunL, YugL, and XH performed data analysis. TL, CZ, SL, and XL drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.
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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.987073/full#supplementary-material

References

Abe, A., Kosugi, S., Yoshida, K., Natsume, S., Takagi, H., Kanzaki, H., et al. (2012). Genome sequencing reveals agronomically important loci in rice using MutMap. Nat. Biotechnol. 30, 174–178. doi: 10.1038/nbt.2095

Bernard, R. (1971). Two major genes for time of flowering and maturity in soybeans. Crop Sci. 11, 242–244. doi: 10.2135/cropsci1971.0011183X001100020022x
Bonato, E. R., and Vello, N. A. (1999). J6, a dominant gene conditioning early flowering and maturity in soybeans. Genet. Mol. Biol. 22, 229–232. doi: 10.1590/s1415-475719990002016

Busteed, R. I. (1971). Inheritance of a soybean flowering response to fluorescent-daylength conditions. Can. J. Genet. Cytol. 13, 783–797. doi:10.1139/g71-180

Cai, Y. P., Wang, L. W., Chen, L., Wu, T. T., Liu, L. P., Sun, S. et al. (2020). Mutagenesis of GmFT2a and GmFT5a mediated by CRISPR/Cas9 contributes for expanding the regional adaptability of soybean. Plant Biotechnol. J. 18, 298–309. doi:10.1111/pbi.13491

Cao, D., Takeshima, R., Zhao, C., Liu, B. H., Jun, A., and Kong, F. J. (2017). Molecular bases of flowering under long days and stem growth habit in soybean. J. Exp. Bot. 68, 1873–1884. doi:10.1093/JEB/ERV394

Carpentieri-Pippolo, V., Alves de Almeida, L., and de Souza Kihl, R. A. (2002). Inheritance of a long juvenile period under short-day conditions in soybean. Genet. Mol. Biol. 25, 463–469. doi:10.1590/S1415-47572002040000016

Chen, L. Y., Nan, H. Y., Kong, L. P., Yue, L., Yang, H., Zhao, Q. S. et al. (2020). Soybean AP1 homologs control flowering time and plant height. J. Integr. Plant Biol. 62, 1292–1298. doi:10.1111/jipb.12988

Choi, I. Y., Hyten, D. L., Matukumalli, L. K., Song, Q. J., Chakry, J. M., Quigley, C. V. et al. (2007). A soybean transcript map gene distribution, haplotype and single-nucleotide polymorphism analysis. Genetics. 176, 685–696. doi:10.1534/genetics.107.070821

Churchill, G. A., and Doerge, R. (1994). Empirical threshold values for quantitative trait mapping. Genetics. 138, 963–971. doi:10.1093/genetics/138.3.963

Cober, E. R., Molnar, S. J., Charette, M., and Voldeng, H. D. (2010). A new locus for early maturity in soybean. Crop Sci. 50, 524–527. doi:10.2135/cropsci2009.04.0174

Cober, E. R., and Morrison, M. J. (2010). Regulation of seed yield and agronomic characters by photoperiod sensitivity and growth habit genes in soybean. Theor. Appl. Genet. 121, 1125–1130. doi:10.1007/s00122-009-1228-6

Cober, E. R., and Voldeng, H. D. (2001a). A new soybean maturity and photoperiod-sensitivity locus linked to El. Crop Sci. 41, 698–701. doi:10.2135/cropsci2001.413689x

Cober, E. R., and Voldeng, H. D. (2001b). Low R:FR light quality delays flowering of EF7 soybean lines. Crop Sci. 41, 1823–1826. doi:10.2135/cropsci2001.2001

Diestro, D., Carpentieri-Pippolo, V., de Souza Kihl, R. A., and Alves de Almeida, L. (2001). Photoperiodism and genetic control of the long juvenile period in soybean. a review. Crop. Breed. Appl. Biotech. 1, 72–92. doi:10.1308/1984–7033-v0101a10

Dong, L. D., Cheng, Q., Fang, C., Kong, L. P., Yang, H., Hou, Z. H. et al. (2012). Parallel selection of distinct Teos alleles drove the adaptation of cultivated and wild soybean to high latitudes. Mol. Plant 15, 308–321. doi:10.1090/MP.2012.10.0014

Dong, L. D., Fang, C., Cheng, Q., Su, T., Kou, K., Kong, L. P. et al. (2021). Genetic basis and adaptation trajectory of soybean from its temperate origin to tropics. Nat. Commun. 12, 5445. doi:10.1038/s41467-021-25800-3

Fang, C., Chen, L. Y., Nan, H. Y., Kong, L. P., Li, Y., Zhang, H. Y. et al. (2019). Rapid identification of consistent novel QTLs underlying long-juvenile trait in soybean by multiple genetic populations and genotyping-by-sequencing. Mol. Breeding. 38, 80. doi:10.1007/s11032-019-0979-2

Fang, C., Liu, J., Zhang, T., Su, T., Li, S. C., Yang, Y. Q., Lv, T. X., Su, T., Chen, L. Y., et al. (2021). The robust CRISPR/Cas9 system for convenient, high-efficiency genome editing in monocot and dicot plants. Mol. Plant 8, 1274–1284. doi:10.1093/molp/jmd054.2021.04.007

McBlain, B. A., and Bernard, R. L. (1987). A new gene affecting the time of flowering in soybeans. Crop Sci. 27, 1006. doi:10.2135/1987cropsci1995.0011183x003500040012x

Michelmore, R. W., Parlan, I., and Kesseli, R. V. (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis - a rapid method to detect markers in specific generic regions by using segregating populations. Proc. Natl. Acad. Sci. U. S. A. 88, 9828–9832. doi:10.1073/pnas.88.21.9828

Mansur, L. M., Orf, J., and Lark, K. G. (1993). Determining the linkage of quantitative trait loci to RFLP markers using extreme phenotypes of recombinant inbreds of soybean (Glycine max L. merr.). Theor. Appl. Genet. 86, 914–918. doi:10.1007/BF00210141

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., et al. (2010). The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1260–1263. doi:10.1101/gr.107524.110

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., et al. (2010). The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1260–1263. doi:10.1101/gr.107524.110

Neumann, N., and James, A. T. (1993). Exploiting the long juvenile trait to improve adaptation of soybeans to the tropics. ACTAR Food Legume Newsletter. 18, 12–14.

Parver, A. Q., and Gardner, F. P. (1987). Daylength and sowing date responses of soybean lines with “juvenile” trait. Crop Sci. 27, 305–310. doi:10.2135/cropsci1997.0011183x007200020037x

Ray, J. D., Himon, K., Mankone, J. E. R., and Malo, M. F. (1995). Genetic control of a long juvenile trait in soybean (Glycine max L. Merr.). Crop Sci. 35, 1001–1006. doi:10.2135/cropsci1995.0011183x003500040012x
Samanfar, B., Molnar, S. J., Charette, M., Schoenrock, A., Dehne, F., Golshani, A., et al. (2017). Mapping and identification of a potential candidate gene for a novel maturity locus, E10, in soybean. *Theor. Appl. Genet.* 130, 377–390. doi: 10.1007/s00122-016-2819-7

Schneeberger, K., and Weigel, D. (2011). Fast-forward genetics enabled by new sequencing technologies. *Trends Plant Sci.* 16, 282–288. doi: 10.1016/j.tplants.2011.02.006

Sinclair, T. R., and Hinson, K. (1992). Soybean flowering in response to the long-jvenile trait. *Crop Sci.* 32, 1242–1248. doi:10.2135/cropsci1992.0011183X003200050036x

Takagi, H., Abe, A., Yoshida, K., Kosugi, S., Natsumi, S., Mitsuoka, C., et al. (2013a). QTL-seq: rapid mapping of quantitative trait loci in rice by whole genome resequencing of DNA from two bulked populations. *Plant J.* 74, 174–183. doi:10.1111/tpj.12105

Takagi, H., Uemura, A., Yaegashi, H., Tamiru, M., Abe, A., Mitsuoka, C., et al. (2013). MutMap-gap: whole-genome resequencing of mutant F2 progeny bulk combined with de novo assembly of gap regions identifies the rice blast resistance gene *pii*. *New Phytol.* 200, 276–283. doi:10.1111/nph.12369

Van Ooijen, J. W. (2004). *MapQTL5*, software for the mapping of quantitative trait loci in experimental populations (Wageningen, Netherlands: Kyazma, B.V.).

Voorrips, R. E. (2002). MapChart: software for graphical presentation of linkage maps and QTLs. *J. Hered.* 93, 77–78. doi:10.1093/jhered/93.1.77

Wang, J., Chu, S., Zhang, H., Zhu, Y., Cheng, H., and Yu, D. (2016). Development and application of a novel genome-wide SNP array reveals domestication history in soybean. *Sci. Rep.* 6, 20728. doi:10.1038/srep20728

Wang, L. S., Fang, C., Liu, J., Zhang, T., Kou, K., Su, T., et al. (2020). Identification of major QTLs for flowering and maturity in soybean by genotyping-by-sequencing analysis. *Mol Breeding.* 40, 99. doi:10.1007/s11032-020-01178-w

Wang, F., Nan, H., Chen, L., Fang, C., Zhang, H., Su, T., et al. (2019). A new dominant locus, E11, controls early flowering time and maturity in soybean. *Mol. Breeding.* 39, 70. doi: 10.1007/s11032-019-0978-3

Watanabe, S., Hideshima, R., Xia, Z., Tsukobu, Y., Sato, S., Nakamoto, Y., et al. (2009). Map-based cloning of the gene associated with the soybean maturity locus E3. *Genetics.* 182, 1251–1262. doi:10.1534/genetics.108.098772

Watanabe, S., Xia, Z., Hideshima, R., Tsukobu, Y., Sato, S., Yamanaka, N., et al. (2011). A map-based cloning strategy employing a residual heterozygous line reveals that the GIGANTEA gene is involved in soybean maturity and flowering. *Genetics.* 188, 395–407. doi:10.1534/genetics.110.125062

Whipple, C. J., Kebrom, T. H., Weber, A. L., Yang, F., Hall, D., Meeley, R., et al. (2011). *Grassy tillers* promotes apical dominance in maize and responds to shade signals in the grasses. *Proc. Natl. Acad. Sci. U. S. A.* 108, E506–E512. doi: 10.1073/pnas.1102819108

Xia, Z., Watanabe, S., Yamada, T., Tsukobu, Y., Nakashima, H., Zhai, H., et al. (2012). Positional cloning and characterization reveal the molecular basis for soybean maturity locus E1 that regulates photoperiodic flowering. *Proc. Natl. Acad. Sci. U. S. A.* 109, E2155–E2164. doi:10.1073/pnas.1117982109

Yi, B., Chen, Y. N., Lei, S. L., Tu, J. X., and Fu, T. D. (2006). Fine mapping of the recessive genic male-sterile gene (*Bnms1*) in *Brassica napus* l. *Theor. Appl. Genet.* 113, 643–650. doi:10.1007/s00122-006-0328-9

Yue, L., Li, X. M., Fang, C., Chen, L. Y., Yang, H., Yang, J., et al. (2021). FTSs interfere with the D1-AP1 feedback loop to control flowering time and shoot determinacy in soybean. *J. Integr. Plant Biol.* 63, 1004–1028. doi:10.1111/jipb.13070

Zhao, C., Takeshima, R., Zhu, J., Xu, M., Sato, M., Watanabe, S., et al. (2016). A recessive allele for delayed flowering at the soybean maturity locus E9 is a leaky allele of FT2a, a FLOWERING LOCUS T ortholog. *BMC Plant Biol.* 16, 20. doi: 10.1186/s12870-016-0704-9

Zhong, J. S., and Kong, F. J. (2022). The control of compound inflorescences: insights from grasses and legumes. *Trends Plant Sci.* 27, 564–576. doi: 10.1016/j.tplants.2021.12.002