Soybean adaption to high-latitude regions is associated with natural variations of GmFT2b, an ortholog of FLOWERING LOCUS T

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ABSTRACT
Day length has an important influence on flowering and growth habit in many plant species. In crops such as soybean, photoperiod sensitivity determines the geographical range over which a given cultivar can grow and flower. The soybean genome contains ~10 genes homologous to FT, a central regulator of flowering from Arabidopsis thaliana. However, the precise roles of these soybean FTs are not clearly. Here we show that one such gene, GmFT2b, promotes flowering under long-days (LDs). Overexpression of GmFT2b upregulates expression of flowering-related genes which are important in regulating flowering time. We propose a ‘weight’ model for soybean flowering under short-day (SD) and LD conditions. Furthermore, we examine GmFT2b sequences in 195 soybean cultivars, as well as flowering phenotypes, geographical distributions and maturity groups. We found that Hap3, a major GmFT2b haplotype, is associated with significantly earlier flowering at higher latitudes. We anticipate our assay to provide important resources for the genetic improvement of soybean, including new germplasm for soybean breeding, and also increase our understanding of functional diversity in the soybean FT gene family.

KEYWORDS
flowering promoter, gene haplotype, Glycine max (L.) Merr., GmFT2b, photoperiod, Soybean

1 | INTRODUCTION

Flowering time is an important trait that regulates plant adaptability and yield. Plants integrate flowering signals from a range of different internal and external cues to transition from vegetative growth to flowering to set seeds. In Arabidopsis, FLOWERING LOCUS T (FT) has emerged as a key integrator of multiple flowering pathways, and the FT protein is now widely accepted as being the proposed flowering hormone florigen itself or, alternatively, as the major component of a more complex florigen signal (Turck, Fornara, & Coupland, 2008; Zeevaart, 2006; Zeevaart, 2008). The molecular mechanisms of FT signalling have been elucidated primarily in Arabidopsis (Abe et al., 2005; Corbesier et al., 2007; Jaeger &
Wigge, 2007; Mathieu, Warthmann, Kütter, & Schmid, 2007), and the prevailing model of FT action has been supported by studies in rice, pumpkin, Populus and several other plant species (Böhlenius et al., 2006; Kojima et al., 2002; Lin et al., 2007; Tamaki, Matsuoka, Wong, Yokoi, & Shimamoto, 2007; Nishikawa et al., 2007; Varkonyi-Gasic et al., 2013; Li, Li, et al., 2013). The expression of FT in leaves is regulated by CONSTANS, a zinc-finger transcription factor, in Arabidopsis (Abe et al., 2005; Wigge et al., 2005); the FT protein is produced in the leaves and transported in the phloem to the shoot apical meristem, where it acts to initiate flowering (Corbesier et al., 2007; Jaeger & Wigge, 2007). It has been suggested that FT and the bZIP-type transcription factor FD may play a promotive role in regulating the expression of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) and FRUITFUL (FUL), and as a result, the FT/FD complex also upregulates the expression of floral meristem identity genes such as APETALA1 (API) and LEAFY (LFY) in Arabidopsis (Benlloch et al., 2011; Kaufmann et al., 2010; Li, Zhou, et al., 2013; Wagner & Meyerowitz, 2010; Weigel, Alvarez, Smyth, Yanofsky, & Meyerowitz, 2007; Mathieu, Warthmann, Küttner, & Schmid, 2007), and the Popolus, pea, cotton, and alfalfa, have been expressed in heterologous expression of GmFT1a in Arabidopsis (Tamaki et al., 2007).

In this study, we isolated a soybean FT homolog, GmFT2b, that is highly homologous to GmFT2a. We obtained transgenic overexpressing lines and also produced lines carrying mutations in GmFT2b using CRISPR/Cas9. Overexpression of GmFT2b promoted flowering under LD conditions, and ft2b mutants showed delayed flowering only under LD conditions. The flowering times of the GmFT2b-ox and the ft2b mutant plants were almost the same as for WT plants under SD conditions. By analysing the expression patterns of flowering-related genes in the GmFT2b-ox and ft2b mutant plants, we inferred that only the flowering-related genes in which expression was up- or down-regulated sufficiently under LD conditions can regulate the flowering time. Haplo-type and phenotypic analysis of GmFT2b indicated that Hap3 is mainly present in cultivars in MGs 0–2, which show earlier flowering times. The results of our study contribute important genetic information and provide tools for the genetic improvement of soybean such as new germplasm for soybean breeding, especially in high-latitude regions.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials and growth conditions

The soybean cultivars 'Zigongdongdou' ('ZGDD') and 'Jack' were used in the present study. The cultivar 'ZGDD' was used for gene cloning. The cultivar 'Jack' was used for Agrobacterium-mediated transformation. Soybean seeds were germinated and the seedlings grown in a controlled culture room at 28°C under LD (16 hr light/8 hr dark) and SD (12 hr light/12 hr dark) conditions.

### 2.2 | GmFT2b cDNA cloning

Total RNA was extracted using Trizol reagent from the trifoliate leaves of soybean cv. 'ZGDD' seedlings. First-strand cDNA was synthesized with Superscript II reverse transcriptase (TransGen Biotech, Beijing, China) and used as a template for further GmFT2b cDNA cloning. Amplification was performed via PCR using KOD-plus-Neo DNA polymerase (Toyobo, Tokyo, Japan). The sequences of the primers used for amplifying the full-length GmFT2b cDNA are given in Table S1.

### 2.3 | Subcellular localization of GmFT2b

The open reading frame (ORF) of GmFT2b was fused with the 5’ end of the GFP gene sequence in a construct under control of the CaMV 35S promoter. The GmFT2b gene was cloned into the p16318 plasmid...
The recombinant fusion plasmids were introduced into onion epidermal cells by particle bombardment using a CaMV 35S:eGFP vector as control. Transformation was achieved with a PDS 1000/He device (BioRad, Hercules, CA, USA), with a 6 cm shot distance, 25 mmHg vacuum and 1,100 psi rupture disc pressure. Green fluorescent protein (GFP) fluorescence was monitored using a Zeiss LSM710 confocal microscope (Carl Zeiss, OKO, Germany).

2.4 | Gene expression analysis

Quantitative RT-PCR (qRT-PCR) was performed using an ABI QuantStudio™ 7 flex Real-Time PCR System (Applied Biosystems). To examine the expression of flowering-related genes in the leaf and shoot apex, these tissues were sampled at 15 days after emergence (DAE) under SD conditions and 30 DAE under LD conditions. Three biological replicates were analysed, with technical replicates for each of the three biological samples. The relative expression levels were analysed using the 2−ΔΔCt method (Livak & Schmittgen, 2001). The gene IDs and primers used to amplify each gene and the internal reference are listed in Table S1. Statistical analyses were performed using Microsoft Excel. The two asterisks represent significant differences at p < .01. The one asterisk represents significant differences at p < .05.

2.5 | Construction of GmFT2b-ox and GmFT2b-CRISPR plasmids

For the overexpression construct (GmFT2b-ox), the CDS of GmFT2b was inserted into the PTF101 vector containing a CaMV 35S promoter and a bar gene (Paz, Martinez, Kalvig, Fonger, & Wang, 2006). For construction of the CRISPR/Cas9 expression vector, a 20-bp sgRNA sequence was designed using the web tool CRISPR-P (http://cbi.hzau.edu.cn/crispr/), and its expression was driven by the Arabidopsis U6 gene promoter. The Cas9 sequence was inserted downstream of the CaMV 2X 35S promoter. The bar gene driven by a CaMV 35S promoter was used as a screening marker. A pair of DNA oligonucleotides for the sgRNA were synthesized by TSINGKE (Beijing) and annealed to generate a double-stranded sgRNA, which was subsequently inserted into the CRISPR/Cas9 expression vector (Cai et al., 2019).

2.6 | Soybean transformation and mutant detection

The overexpression vector (GmFT2b-ox) and CRISPR/Cas9 expression vector (GmFT2b-CRISPR) plasmids were transformed into Agrobacterium tumefaciens strains EHA101 and EHA105 via electroporation, respectively. The soybean cultivar ‘Jack’ was used for tissue culture and transformation according to a previously-published protocol (Chen et al., 2018).

The GmFT2b-overexpressing transgenic plants were screened by PCR and LibertyLink strip detection. The LibertyLink strips were used to determine the presence of the PAT protein in the transgenic plants. The potential GmFT2b mutants were then screened by DNA sequencing analysis. Briefly, genomic DNA was extracted from the leaves of each individual plant in the T0 generation, and the regions spanning the target sites were amplified by PCR using Phanta® Super Fidelity DNA Polymerase (Vazyme Biotech) and sequenced. Different types of gene editing events can be identified by DNA sequencing. Short base insertions or deletions (not in multiples of three) induced by CRISPR/ Cas9 can lead to translational frameshift mutations. DNA from plants that were heterozygous for the mutations showed overlapping peaks on the sequencing chromatograms from the target sites to the end of the DNA fragment. The wild-type and homozygous mutants had no overlapping peaks at the target sites. The homozygous mutant types were identified by sequence alignment against the wild-type gene sequence (Cai et al., 2019).

2.7 | Flowering time measurements and statistical analyses

The flowering time of each soybean plant was recorded as days from emergence to the R1 stage (the time at which the first flower appears at any node on the main stem; Fehr & Caviness, 1977). For quantitative analyses of flowering time, individual soybean plants were analysed for each genotype. Statistical analyses were performed using Microsoft Excel, and the data was analysed by ANOVA. The two asterisks represent significant differences at p < .01.

3 | RESULTS

3.1 | Cloning of GmFT2b and subcellular localization of the protein

The GmFT2b gene was isolated from ‘ZGDD’, a photoperiod-sensitive soybean cultivar. GmFT2b contains a 531-bp ORF that encodes a predicted protein of 176 amino acids. The genomic sequence of GmFT2b spans 3,014 bp and contains three introns and four exons. GmFT2b (Glyma.16g151000) is located on soybean chromosome 16 and is close to GmFT2a (Glyma.16g150700). GmFT2b shares 90.91% amino acid sequence identity with its paralog GmFT2a (Figure S1).

To determine the subcellular localization of the GmFT2b protein in planta, we fused the GmFT2b coding region with the gene for green fluorescent protein (GFP) under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter and transfected the resulting p35S::GmFT2b-GFP plasmid into onion epidermal cells. Confocal microscopy showed that the GmFT2b protein is expressed in the cytoplasm and nucleus, with a stronger signal in the nucleus (Figure S2), similar to the cellular localization of the
other functional phosphatidylethanolamine binding proteins in soybean (Wang et al., 2015).

3.2 | Overexpression of GmFT2b in soybean promotes flowering under LD conditions

To investigate the function of GmFT2b, a construct containing the GmFT2b CDS driven by the CaMV 35S promoter was transformed into the mid-maturing group 2 soybean cv. ‘Jack’. The positive transgenic soybean plants were detected by PCR and LibertyLink strips (Figure S3). PCR was used to check for the presence of the PAT protein in transgenic plants. Under LD conditions, the WT plants and the LibertyLink strips were used to determine the presence of the identified.

To investigate the function of GmFT2b, the overexpression of FT-related genes under SD and LD conditions in soybean; these include GmFT2a, GmFT3a, GmFT4 and GmFT5a. GmAP genes (GmAP1a, GmAP1b and GmAP1c), GmSOC genes (GmSOC1a and GmSOC1b), GmFUL, GmLFY, GmAG.

In the GmFT2b-ox plants, we found that the expression of GmFT genes including GmFT2a and GmFT5a were significantly upregulated in leaves, GmFT4 was unchanged under LD conditions. The expression levels of GmAP1 (a, b and c), GmSOC1 (a and b), GmFULa, GmLFY2 and GmAG were slight upregulated in the shoot apex under LD conditions. Under SD conditions, there was no significant change in the expression of GmFT2a, GmFT5a, GmFT1a, GmFT3a and GmFT4 in leaves. The expression levels of GmAP1 (a and c), GmSOC1 (a and b), GmLFY2 and GmAG were not affected (Figure 4). In f2b CRISPR mutants, the expression levels of GmFT1a, GmFT2a, GmFT3a, GmFT4, GmFT5a,
GmAP1 (a, b and c), GmSOC1 (a and b), GmFULa, GmLFY2 and GmAG showed no marked change under LD and SD conditions (Figure 5).  

3.5 Analysis of GmFT2b haplotypes revealed their distribution in soybean varieties in the different maturity groups

We investigated the nucleotide polymorphisms in the coding region of GmFT2b in 195 soybean accessions with varied flowering times using whole-genome resequencing. We found single-base changes at three sites in the fourth exon of GmFT2b. No predicted frameshift mutations or premature stop codons were found in the coding regions. The base changes in the fourth exon did not change the amino acid sequence and protein. The four major haplotypes with higher frequencies were identified and chosen for analysis (Figure 6a). The haplotypes of the GmFT2b promoter were also for analysis (Figure S4). Four haplotypes were identified in GmFT2b promoter, which mainly corresponded to the four haplotypes in the coding region of GmFT2b. We further investigated the maturity groups...
of the soybean varieties that carry the major GmFT2b haplotypes. Hap3 was mainly distributed in the varieties belonging to MGs 0, 1 and 2. Hap2 was mainly distributed in varieties in MG 2–MG 4, and Hap1 was mainly found in varieties in MGs 3 and 4. Hap4 was found to be dispersed across the various maturity groups (Figure 6b).
3.6 Distribution of major GmFT2b haplotypes in soybean varieties from diverse geographical origins

We compared flowering times in the six environments (Heihe, Changchun, Beijing, Xinxiang, Hunan, Sanya) for varieties carrying different GmFT2b haplotypes (Figure 7). The flowering times were examined in regions at six different latitudes in China; from north to south these are Heihe (N50°15', E127°27'), Changchun (N43°49', E125°21'), Beijing (N40°09', E116°14'), Xinxiang (N35°18', E113°55'), Hunan (N27°49', E112°56') and Sanya (N18°21', E109°10'). We found that the Hap3 varieties showed earlier flowering times, while Hap1 and Hap4 varieties flowered later. A majority of the varieties carrying...
GmFT2b haplotypes Hap1 and Hap4 were unable to flower in Heihe due to the high latitude (N50').

We also analyzed the geographical distribution of varieties with major GmFT2b haplotypes. Hap1 was present in a comparatively wide distribution in the southern and middle region of China. Hap2 was mostly found at higher latitude regions in the central and northern parts of the country. Hap3 occurred at higher latitudes in the northern regions. The geographical distribution of Hap4 was in the southern parts of China at low latitudes (Figure S5). Notably, all of the varieties with Hap3 can flower in the northern latitudes, which suggests that the Hap3 genotype may contribute to early flowering at high latitudes.

4 | DISCUSSION

Soybean is a diploid species that evolved from an ancient tetraploid, and its genome has undergone homologous chromosomal recombination and reassortment of the entire genome during its long evolutionary history (Wang et al., 2015). At present, it has been shown that the soybean genome encodes at least ten members of the GmFT gene family. Several studies have demonstrated that GmFT orthologs have different temporal and spatial expression patterns as well as different functions (Endo et al., 2005; Kotoda et al., 2010). Further studies of the GmFT2b and other GmFT genes may reveal novel molecular mechanisms that act to regulate flowering in soybean.

We found that GmFT2b-ox plants exhibited an early-flowering phenotype under non-inductive conditions (LD), while ft2b mutants exhibited a later flowering phenotype than did WT plants under LD conditions. Surprisingly, the flowering times of GmFT2b-ox and ft2b mutant plants were almost the same as WT plants under SD conditions. We examined the expression patterns of some GmFT homolog genes and several flowering-related genes that respond to the overexpression of GmFT2b in Gmft2b-ox and ft2b mutant plants in leaves and the shoot apex. We found that overexpression of GmFT2b upregulated the expression of GmFT2a and GmFT5a under LD conditions in the GmFT2b-ox plants, the expression of three GmAP1 orthologs (GmAP1a, GmAP1b, GmAP1c), GmSOC1b, GmFULa, GmLFY2 and GmAG were slightly upregulated. The gene expression levels have no marked changes induced by SD. We inferred that early flowering may require higher expression levels of flowering-related genes. In ft2b mutants, flowering time was delayed only under LD conditions, and the expression of almost the tested genes was slight lower than in the WT under LD conditions. Under SDs, except GmFT2b, the expression of other genes showed no marked change. Taken together, our results suggest that GmFT2b can influence the expression of GmFT genes and other flowering-related genes under LD conditions in soybean. The differences in the expression levels between LDs and
SDs leads to the different flowering phenotypes. Only the flowering-related genes in which expression is up- or downregulated to a sufficient degree under LDs can regulate flowering time.

Based on the results of the present study and previous reports (Liu et al., 2018; Lu et al., 2017; Yue et al., 2017), we propose a ‘weight’ model for soybean flowering under SD and LD conditions (Figure 8). In this model, we consider that the transformation from vegetative growth to reproductive growth is due to the balance of flowering activators and FIs. The FIs in SDs may be less than in LDs. Under SD conditions, the inhibition of E1 on the expression of GmFT genes is relieved by the J gene (Lu et al., 2017; Yue et al., 2017). Overexpression of GmFT2a promotes early flowering under SD conditions, but overexpression of GmFT5a or GmFT2b does not change flowering time (Cai et al., 2019). The floral activator GmFT2a is more important than GmFT5a and GmFT2b under SD conditions. GmFT2a is sufficient to overcome the effect of FIs to promote the transformation to reproductive growth. The effects of GmFT5a and GmFT2b are less obvious. Under LD conditions, the FT genes are inhibited by E1. More flowering activators are required to overcome the increased number of FIs. All of the GmFT2a-ox, GmFT2b-ox, or GmFT5a-ox plants will flower under LD conditions (Cai et al., 2019). The ft5a mutants showed much later flowering compared to the ft2a and ft2b mutants (Cai et al., 2019). Of these, GmFT5a is more important than GmFT2a and GmFT2b under LD conditions.

By investigating the nucleotide polymorphisms in the GmFT2b coding region in 195 soybean accessions, we found that the GmFT2b haplotypes are associated with flowering time. Soybean varieties carrying GmFT2b haplotype Hap3 exhibited significantly early flowering in all six environments. Most varieties with the Hap1 or Hap4 haplotypes were unable to flower normally when they were grown at Heihe (higher latitude). In addition, the geographical distribution and MG
distribution of the GmFT2b haplotypes showed that Hap3 is only found in varieties grown in higher latitude regions in northeast China that belong to earlier maturing varieties from MGs 0–2. Hap4 is found in varieties grown in lower latitude regions in southern China that belong to later maturing varieties in MGs 000–8. Our previous studies showed GmFT2a also has four major haplotypes with higher frequencies (Cai et al., 2019). FT2a-Hap1 was mostly found in the Huanghuaihai. FT2a-Hap2 was mostly found in higher latitude region in the north and the Huanghuaihai. FT2a-Hap3 was present in the south. FT2a-Hap4 was comparatively wide, but was rare in the northeast. FT2a-Hap2 was mainly distributed in the varieties of MG 1, MG 2 and MG 3. The FT2a-Hap1, FT2a-Hap3 and FT2a-Hap4 genotypes were not found among the earlier maturing varieties (MG 000, MG 00 and MG 0). These results suggest that different GmFT2a and GmFT2b haplotypes have considerable effects on the diversity of flowering time in soybean at different latitudes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

L.C., Y.C. and M.Q. performed the experiments. L.C. and W.H. wrote the manuscript. L.W. T.W. and L.L. provided the data for the loci in the 195 soybean accessions of the diversity panel. W.Y. assisted in soybean transformation. S.S. and C.W. provided soybean varieties. S.Y. and B.J. participated in some experiments. W.H. and T.H. designed and advised on the experiments and revised the manuscript.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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