Received: 30 March 2023 | Revised: 25 September 2023 | Accepted: 5 October 2023

DOI: 10.1111/pce.14739

ORIGINAL ARTICLE

Plant, Cell & Environment | WILEY

The *FLOWERING LOCUS T 5b* positively regulates photoperiodic flowering and improves the geographical adaptation of soybean

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Funding information
Major Science and Technology Projects of China, Grant/Award Number: 2016ZX08010-004; National Natural Science Foundation of China, Grant/Award Number: 31871644; Ministry of Science and Technology of China, Grant/Award Number: 2016YFD0100504; CAAS (Chinese Academy of Agriculture Sciences) Agricultural Science and Technology Innovation Project

Abstract

Plants can sense the photoperiod to flower at the right time. As a sensitive short-day crop, soybean (*Glycine max*) flowering varies greatly depending on photoperiods, affecting yields. Adaptive changes in soybeans rely on variable genetic loci such as *E1* and *FLOWERING LOCUS T* orthologs. However, the precise coordination and control of these molecular components remain largely unknown. In this study, we demonstrate that *GmFT5b* functions as a crucial factor for soybean flowering. Overexpressed or mutated *GmFT5b* resulted in significantly early or later flowering, altering expression profiles for several downstream flowering-related genes under a long-day photoperiod. GmFT5b interacts with the transcription factor GmFDL15, suggesting transcriptional tuning of flowering time regulatory genes via the GmFT5b/GmFDL15 complex. Notably, *GmFT5a* partially compensated for *GmFT5b* function, as *ft5a ft5b* double mutants exhibited an enhanced late-flowering phenotype. Association mapping revealed that *GmFT5b* was associated with flowering time, maturity, and geographical distribution of soybean accessions, all associated with the *E1* locus. Therefore, *GmFT5b* is a valuable target for enhancing regional adaptability. Natural variants or multiple mutants in this region can be utilized to generate optimized soybean varieties with precise flowering times.

KEYWORDS
CRISPR/Cas9, *GmFT5b*, regional adaptability

1 | INTRODUCTION

Soybean (*Glycine max*) is a short-day (SD) plant and is highly influenced by day length for timely flowering, which is essential for stable yields (Cai et al., 2018; Lu et al., 2020). However, this photoperiodic response restricts soybean cultivation to specific regions, making introducing and domesticating varieties challenging (Lin et al., 2020; Lu et al., 2017). To address this, understanding the regulatory mechanisms governing flowering in response to photoperiod is crucial. Identifying molecular targets through this exploration could result in the improved adaptability of soybean varieties to environments with varied day lengths.

Numerous genetic loci are essential in determining soybean flowering, enabling precise maturity and adaptation (Lin et al., 2020;

Qiang Su, Li Chen, and Yupeng Cai contributed equally to this work.

Xu et al., 2021). Some loci like *E1–E4*, *E7*, *E8*, *E10*, *PRR37*, and *Tof11/Tof12* inhibit flowering, while *E6*, *E9*, *E11*, and *J* induce early flowering in soybean (Cober, 2011; Cober et al., 2010; Cober & Voldeng, 2001; Lin et al., 2020; Liu et al., 2008; Lu et al., 2017; Samanfar et al., 2017; Wang et al., 2019, 2020, 2023; Watanabe et al., 2009; Xia et al., 2012; Zhao et al., 2016). The soybean genome contains at least 11 *FT* gene homologs that contribute significantly to flowering regulation (Kong et al., 2010; Lin et al., 2021). Under long-day (LD) conditions, *E1*, a legume-specific flowering repressor, is activated by phytochrome and the circadian clock (Xia et al., 2012). This repressor contains a putative bipartite nuclear localization signal and a region distantly related to the B3 domain and results in the suppression of *FT* gene expression (Lu et al., 2017; Xia et al., 2012). In SD conditions, J (EARLY FLOWERING 3) physically interacts with the GATWCG motifs of the *E1* promoter, reducing its transcriptional level and relieving the inhibition of *FLOWERING LOCUS T 2a/5a*, thereby promoting flowering (Lu et al., 2017). Consistent with *Arabidopsis*, the FT-FD module in soybeans plays a role in regulating flowering, which FT and FD play a directly or indirectly promotive role in the flowering regulatory network, which includes many known genes, such as *AP1*, *FUL*, *SOC1*, and *LFY* (Balanzà et al., 2018; Lee et al., 2008). Unlike *Arabidopsis*, soybean *FTs* undergo functional differentiation under different photoperiods. For instance, *GmFT2a* has a greater impact on flowering under SD conditions, while *GmFT5a* has a stronger effect under LD conditions. The functional divergence of GmFTs is likely influenced by their interactions with different FDs (Takeshima et al., 2019).

In soybeans, flowering time is influenced by natural or engineered variations in critical genes, enabling adaptability to different regions (Cai et al., 2020; Chen et al., 2018; Li et al., 2021; Liu et al., 2020). For instance, the dominant allele *E1* strongly suppresses flowering, but recessive *e1* genotypes (*e1-as*, *e1-fs*, *e1-nl*) promote flowering and expand regional adaptability (Liu et al., 2022; Xia et al., 2012). The *LJ16.1* (*GmFT2a*) and *LJ16.2* (*GmFT5a*) loci confer a long juvenile trait, reducing sensitivity to SD-induced flowering and enabling soybean cultivation in lower latitude regions (Li et al., 2021). CRISPR-edited soybeans with both *GmFT2a* and *GmFT5a* mutations exhibit extremely late flowering, resulting in *ft2a ft5a* double mutants with high yield potential in tropical regions (Cai et al., 2020). A major *GmFT2b* enables earlier flowering and adaptation to higher latitudes (Chen et al., 2018). *GmFT3a* promotes flowering under LD conditions, and the *GmFT3a*-Hap2 haplotype enhances adaptation to high latitudes (Yuan et al., 2022). *GmFT1a* and *GmFT4* are flowering repressors, but research on natural variants' geographic distribution is limited (Liu et al., 2018). GmFT5b, similar to the *Arabidopsis* FLOWERING LOCUS T (AT1G65480), is implicated as a flowering promoter in *Arabidopsis* (Fan et al., 2014; Lee et al., 2021), yet its function and regulatory mechanism in soybean remain poorly understood.

This study aimed to investigate the function and mechanism of *GmFT5b* in soybean flowering regulation. We identified the *GmFT5b* gene, which responds to photoperiod induction. Through over-expression or impairment experiments under different photoperiod conditions, we demonstrated the positive regulatory role of *GmFT5b* in soybean photoperiodic flowering, partly compensated by *GmFT5a*. We discovered a transcription factor, GmFDL15, that specifically interacts with GmFT5b. Transcriptional expression profiling showed that *GmFT5b* regulates the expression of flowering-related genes in a photoperiod-dependent manner. Association mapping revealed that the *GmFT5b* locus is influenced by the *E1* locus, contributing to soybean's regional adaptation by affecting flowering and maturity times. These findings highlight the crucial role of *GmFT5b* in controlling soybean flowering and regional adaptability, offering potential applications for soybean genetic improvement.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

The soybean variety Jack was used as wild-type (WT) *Glycine max* in this study. The *E1* RNAi soybean plants and *E1*-knockout soybean plants were created and conserved in our laboratory. The seeds of WT, *GmFT5b* transgenic overexpression lines (OE), *ft5b*, and *ft5a ft5b* mutant plants were sown in plastic pots, which were placed in a standard LD (16 h of light at 30°C, 8 h of dark at 22°C) or SD (12 h of light at 30°C, 12 h of dark at 22°C) photoperiods in growth chambers and natural long-day (NLD) conditions (from May 22nd [approximately 15 h light and 9 h of dark] to September 20th [approximately 12 h of light and 12 h of dark]) in Beijing, China (39°58′ N, 116°20′ E). The 160 accessions were planted in Sanya (18°21′ N, 109°10′ E), Hunan (27°49′ N, 112°56′ E), Xinxiang (35°08′ N, 113°45′ E), Beijing (40°09′ N, 116°14′ E), Changchun (43°49′ N, 125°21′ E) and Heihe (50°15′ N, 127°27′ E), in China, in 2017.

2.2 | Flowering time measurements and statistical analyses

The flowering time was counted from seedling emergence (VE) to R1 stage (the first flower appears at any node in the main stem), and the maturity time was calculated from seedling emergence to beginning maturity (R7) as previously described (Fehr et al., 1971). At least 15 individual soybean plants were analyzed per genotype. All the statistical analyses were carried out using Microsoft Excel and GraphPad Prism 8 software. The flowering time is shown as the mean value standard deviation.

2.3 | DNA and RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Genomic DNA was extracted with TPS buffer (100 mM Tris-HCl, 10 mM EDTA, 1 M KCl, PH 8.0) as follows. Briefly, approximately 100 mg of soybean leaves were macerated in TPS buffer, and the

supernatant was obtained by centrifugation. Genomic DNA was then extracted by anhydrous alcohol precipitation at −20°C.

For the flowering-related gene expression profiling analysis, various tissues were sampled 7 days before flowering under different photoperiods (Cai et al., 2018). The fully developed trifoliate leaves and shoot apices (containing meristem tissue) of Jack (control), OE2, and *ft5b* mutant plants were sampled at 15 days after emergence (DAE) under SD, 20 DAE under NLD, and 35 DAE under NLD. As previously described, total RNAs of soybean samples were extracted with TRIZOL reagent (Su et al., 2022). Approximately 1 μg total RNA was reverse-transcribed with ReverTra Ace qPCR RT Master Mix with gDNA Remover kit. Quantitative PCR reactions were performed on an ABI QuantStudio™ 7 Flex Real-Time PCR System device using a ChamQ SYBR qPCR Master Mix (Low ROX Premixed) following the manufacturer's protocols. Each qPCR reaction was run with three biological replicates. Relative transcript levels were calculated by the $2^{-\Delta\Delta C_t}$ method, and the soybean *GmActin* (Glyma.18G290800) gene was used as an internal control. The primers for qPCR analysis used in this study are listed in Supporting Information: Table S2.

2.4 | Generation of transgenic and mutant plants

The full-length *GmFT5b* coding sequence was amplified from Jack complementary DNA (cDNA) by primers GmFT5b-FQ-F/R. To generate the pTF101-EGFP-*GmFT5b*- vector, *GmFT5b* was amplified by primer pairs GmFT5b-OX-F/R and then subcloned into the *Xba*I site of the pTF101-EGFP vector with an EGFP epitope tag. *GmFT5b* transgenic lines were generated according to previously reported *Agrobacterium*-mediated transformation protocols (Chen et al., 2018).

To obtain *GmFT5b* loss-of-function frameshift mutants, we specifically knocked out the *GmFT5b* gene in soybeans using the CRISPR/Cas9 system as described previously (Cai et al., 2018). CRISPR-P software (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) was used to design the target site (GmFT5b-SP1) of *GmFT5b*, which was in the first exon of *GmFT5b*. To confirm homozygosity in *ft5b* mutant plants, we used the GmFT5b-612F/R primers to amplify the *GmFT5b* fragment. The 612 bp PCR products were sequenced and confirmed by alignment with the WT sequence. The *ft5a ft5b* mutant plant was generated by hybridizing the *ft5b* plant with the *ft5a* plant as described previously (Cai et al., 2020). The *ft5a ft5b* mutants were examined by Sanger sequencing.

2.5 | Yeast two-hybrid (Y2H) assays

For yeast two-hybrid analysis, The CDSs of *GmFT5b* and *GmFDL15* were amplified from the cDNA of soybean cultivar Jack using primers listed in Supporting Information: Table S2. Then, after confirmation by sequencing, the amplicons of *GmFT5b* were cloned into pGBKT7 (BD as the bait) and *GmFDL15* into pGADT7 (AD as the prey) vectors, resulting in *GmFT5b*-BD *and GmFDL15*-AD, respectively. The bait and prey were cotransformation into *Saccharomyces cerevisiae* AH109 cells following the manufacturer's instructions. Those transformed yeast cells were grown on dropout supplements (SD/-Leu-Trp) and selective medium quadruple dropout supplements (SD/-Leu-Trp-His-Ade) under 30°C for 3 days. Empty pGBKT7 and pGADT7 vectors were used as negative controls.

2.6 | Bimolecular fluorescence complementation (BiFC) assays

The CDS of *GmFT5b* and *GmFDL15* were amplified and cloned into the vector pDONR™221. Then, the GmFT5b-pDONR™221 and GmFDL15-pDONR™221 were recombined into the pEarleyGate 201 and pEarleyGate 202, respectively. For transient expression, the leaves of *Nicotiana benthamiana* were injected with *Agrobacterium tumefaciens* (GV3101 strain), which carried GmFT5b-pEarleyGate 201 and GmFDL15-pEarleyGate 202 together with p19 strain and nuclear marker (NM–mRFP, mCherry). All the infiltrated leaves were observed and imaged by a confocal laser scanning microscope (Olympus Corporation FLUOVIEW FV3000).

2.7 | Total protein extraction and Western blot analysis

GmFT5b overexpression seedlings grown under different photoperiods were sampled at 14 DAE. Following established protocols for plant protein extraction (Su et al., 2022), the total soluble proteins were extracted from *GmFT5b* overexpression soybean (OE2, OE4, and OE5 lines). Subsequently, total soluble proteins were analyzed by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis electrophoresis and then transferred to a 0.45 μm PVDF membrane, incubated with the anti-GFP mouse monoclonal antibodies (ABclonal, 1:3000-fold dilution), and stored overnight at 4°C. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) (TransGen Biotech, 1:5000-fold dilution) at 25°C for 1 h. Finally, the GmFT5b-EGFP fusion proteins were visualized using Clarity Western ECL Substrate Reagent (Bio-Rad) in an Amersham Imager 600 (GE Healthcare).

2.8 | Pull-Down assays

The *GmFT5b* and *GmFDL15* CDSs (with added *BamH*I/*EcoR*I or *EcoR*I/*Sma*I sites, respectively) were amplified by PCR with the primers GmFT5b-MBP-F/R and GmFDL15-GST-F/R (Supporting Information: Table S2). The PCR products were subsequently subcloned into the pMAL-C5X vector and pGEX4T-1, and then confirmed by sequencing. Next, the pGEX4T-1-GmFDL15, pGEX4T-1, pMAL-C5X-GmFT5b, and pMAL-C5X plasmids were each transformed in *Escherichia coli* strain BL21. The BL21 cells were induced with 0.1 mM IPTG for 4 h to obtain fusion proteins in a 120-rpm shaking incubator at 28°C. Cultures were then centrifuged at 4000*g* to

harvest cells, which were lysed by the freeze-thaw method, as previously described (Su et al., 2022).

For pull-down assay, the cell debris with MBP/MBP-GmFT5b fusion proteins were incubated with Amylose Resin (New England Biolabs) at 4°C overnight in phosphate buffer saline (PBS) buffer. Subsequently, the resin was washed five times with PBS buffer and then incubated with 10 μg of GST-GmFDL15 fusion proteins with end-over-end rotation overnight. After incubation, the resin was washed 10 times using PBS buffer and eluted with MBP elution buffer (0.04% maltose solution). Finally, the eluted proteins (10 μL) were analyzed by 10% SDS-PAGE and western blot detection with anti-MBP (ABclonal, 1:3000), anti-GST (ABclonal, 1:10,000), and HRP-conjugated goat anti-mouse IgG (H + L) (TransGen Biotech, 1:5000) antibodies.

2.9 | Haplotype analysis

To investigate the natural variation of *GmFT5b*, we analyzed the re-sequenced data of 160 varieties that were chosen because of their large differences in the ecological region diversity and photoperiod sensitivity. The 160 soybean accessions were planted in six different latitudes in China: Heihe (50°15′ N, 127°27′ E), Changchun (43°49′ N, 125°21′ E), Beijing (40°09′ N, 116°14′ E), Xinxiang (35°18′ N, 113°55′ E), Hunan (27°49′ N, 112°56′ E) and Sanya (18°21′ N, 109°10′ E).

3 | RESULTS

3.1 | *GmFT5b* positively regulates flowering in soybeans under varying photoperiod conditions

GmFT5b is responsive to photoperiod and acts as a flowering promoting in *Arabidopsis* (Fan et al., 2014; Lee et al., 2021). Considering its regulation by photoperiod and circadian rhythm, we hypothesized that *GmFT5b* might also play a crucial role in flowering regulation in soybeans. To explore the impact of photoperiod on soybean flowering, we generated *GmFT5b* overexpression lines through *Agrobacterium*-mediated transformation, utilizing a *CaMV 35S* promoter-driven expression vector carrying *GmFT5b* (Supporting Information: Figure S1A). Three *GmFT5b* transgenic lines (OE2, OE4, and OE5) were confirmed through various assays, including PCR screening, LibertyLink strips, western blot, and qPCR (Supporting Information: Figure S1B-E). The T_2-generation transgenic plants were then subjected to further analysis. Under different photoperiod conditions (SD; 12 h light and 12 h dark), LD (16 h light and 8 h dark), and NLD (May 22–September 20) field conditions in Beijing, China (39°58′ N, 116°20′ E)—we evaluated the growth and flowering characteristics of both transgenic and WT plants.

Under SD conditions, WT soybean plants flowered at 26.6 DAE, while the transgenic plants (OE2, OE4, and OE5) exhibited early flowering phenotypes at 20.5, 25.1, and 26.4 DAE, respectively. Notably, OE2 and OE4 showed significant early flowering by 6.1 and 1.5 days earlier than WT, respectively (Figure 1a,b). Similarly, under NLD conditions, the transgenic lines flowered at 9.7 (OE2), 2.6 (OE4), and 1.6 (OE5) days earlier than WT (28.0 DAE) (Supporting Information: Figure S1C,D). Furthermore, under LD conditions, OE2 (27.1 DAE) exhibited extreme early flowering with floral induction initiating 17.7, 5.1, and 2.4 days earlier than WT plants (44.8 DAE) (Figure 1e,f). Among the transgenic lines, OE2 displayed the strongest effect on flowering induction. These findings, coupled with high *GmFT5b* expression in transgenic plants, indicate that *GmFT5b* positively regulates soybean flowering under different photoperiods, and higher *GmFT5b* expression levels are associated with earlier flowering times.

3.2 | CRISPR/Cas9-edited *ft5b* mutant plants exhibited late flowering

Next, we employed CRISPR/Cas9 genome editing to create *GmFT5b* knockout mutants called *ft5b*. Among 33 independent T_0 transgenic *ft5b* mutant lines screened using PCR and Sanger sequencing, two homozygous *ft5b* mutants (*ft5b*-m1 and *ft5b*-m2) were obtained in T_1 plants, both inducing frameshifts and premature stop codons in *GmFT5b* (Supporting Information: Figure S2A). Specifically, the *ft5b*-m1 mutant had an 8-bp deletion and was classified as a transgene-free type, while the *ft5b*-m2 mutant contained a 1-bp insertion (Supporting Information: Figure S2B-D). All progeny of the homozygous *ft5b*-m1 mutant were also transgene-free homozygous *ft5b*-m1 mutants, as confirmed by LibertyLink strips (Supporting Information: Figure S2E).

Under SD conditions, the average flowering times of two independent *ft5b* mutant plants, 26.3 and 26.1 DAE, were not significantly different from that of the WT, which was 26.6 DAE (Figure 2a,b, Supporting Information: Figure S2H). However, under NLD conditions, the *ft5b* mutant plants exhibited significantly delayed flowering compared to WT, with differences of 2.1 and 1.8 days, respectively (30.1 and 29.8 vs. 28.0 DAE) (Figure 2c,d, Supporting Information: Figure S2F). Similarly, under LD conditions, the flowering dates of the *ft5b* mutants were significantly later than those of WT plants (47.8 and 48.0 vs. 44.8 DAE) (Figure 2e,f, Supporting Information: Figure S2G). These findings strongly suggest that the loss of *ft5b* function results in delayed flowering in soybeans under LD and NLD conditions, but not under SD conditions.

3.3 | *ft5a ft5b* double mutants exhibited late flowering phenotypes

To investigate the genetic compensation response (GCR) in *ft5b*-m1 plants under LD conditions, we assessed the expression of *FT* genes. As expected, *GmFT5b* expression was significantly upregulated in OE2 plants and downregulated in *ft5b*-m1 plants (Supporting Information: Figure S3A). Interestingly, only *GmFT5a* was upregulated considerably in *ft5b*-m1 mutant plants compared to the wild type (Supporting Information: Figure S3A,B). Additionally, *GmFT5a* and

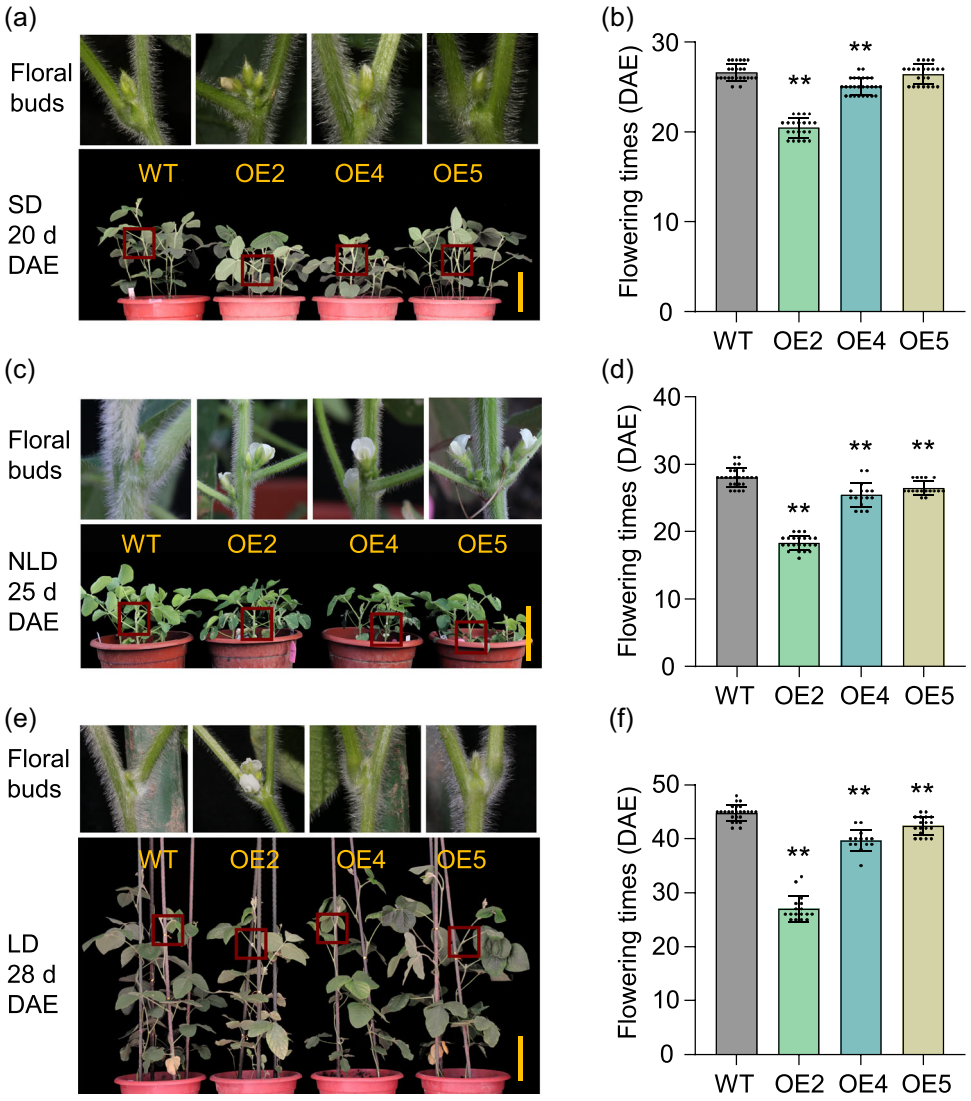

FIGURE 1 Overexpression of *GmFT5b* promotes early flowering in soybeans. Phenotypes of WT and *GmFT5b* overexpression soybean lines (OE2, OE4, OE5) under SD (a), NLD (c) and LD (e) conditions. Flowering times of WT and *GmFT5b* overexpression soybean under SD (b), NLD (d), and LD (f) conditions. The *GmFT5b* overexpression soybean displayed significant early flowering compared to WT (**$p < 0.01$; *$p < 0.05$). Error bars represent standard deviation. Scale bar = 30 cm. DAE, days after emergence; LD, long day; NLD, natural long-day; SD, short-day; WT, wild type.

GmFT5b mutually regulated each other under different photoperiod conditions, particularly in the short apical meristem (SAM) (Supporting Information: Figure S3A,B). To further investigate the combined effect of *GmFT5a* and *GmFT5b*, we generated *ft5a ft5b* double mutants by crossing the *ft5b*-m1 mutant plants (with an 8-bp deletion) with *ft5a* mutant plants (with a 1-bp insertion) and obtained one homozygous *ft5a ft5b* double mutant in the F_2 generation (Supporting Information: Figure S3C). Subsequently, we examined the flowering time of *ft5a ft5b* double mutants under different photoperiod conditions.

Under SD conditions, there were no significant differences in flowering time between the WT and all mutant plants. Both WT and mutant plants (*ft5a*, *ft5b*-m1, and *ft5a ft5b* double mutants) flowered at 26.6, 26.1, 26.3, and 26.8 DAE, respectively (Figure 2a,d,g). However, under LD conditions, the flowering time of *ft5a ft5b* double mutant plants (77.1 DAE) was significantly later than that of WT plants (44.8 DAE), *ft5b*-m1 (47.8 DAE), *ft5b*-m2 (48.0 DAE), and *Gmft5a* mutants (66.8 DAE) (Figure 2c,e,h). These results suggest that the effect of *GmFT5b* on flowering was partially compensated, likely due to the GCR, whereas the *ft5a ft5b* double mutants overcame the GCR and displayed a robust phenotype with delayed flowering under LD conditions.

3.4 | *GmFT5b* regulates transcription levels of flowering-related genes

In OE2 plants, the expression levels of several flowering-related genes, including four *GmAP1* genes, two *GmFUL* genes, *GmLFY1* and

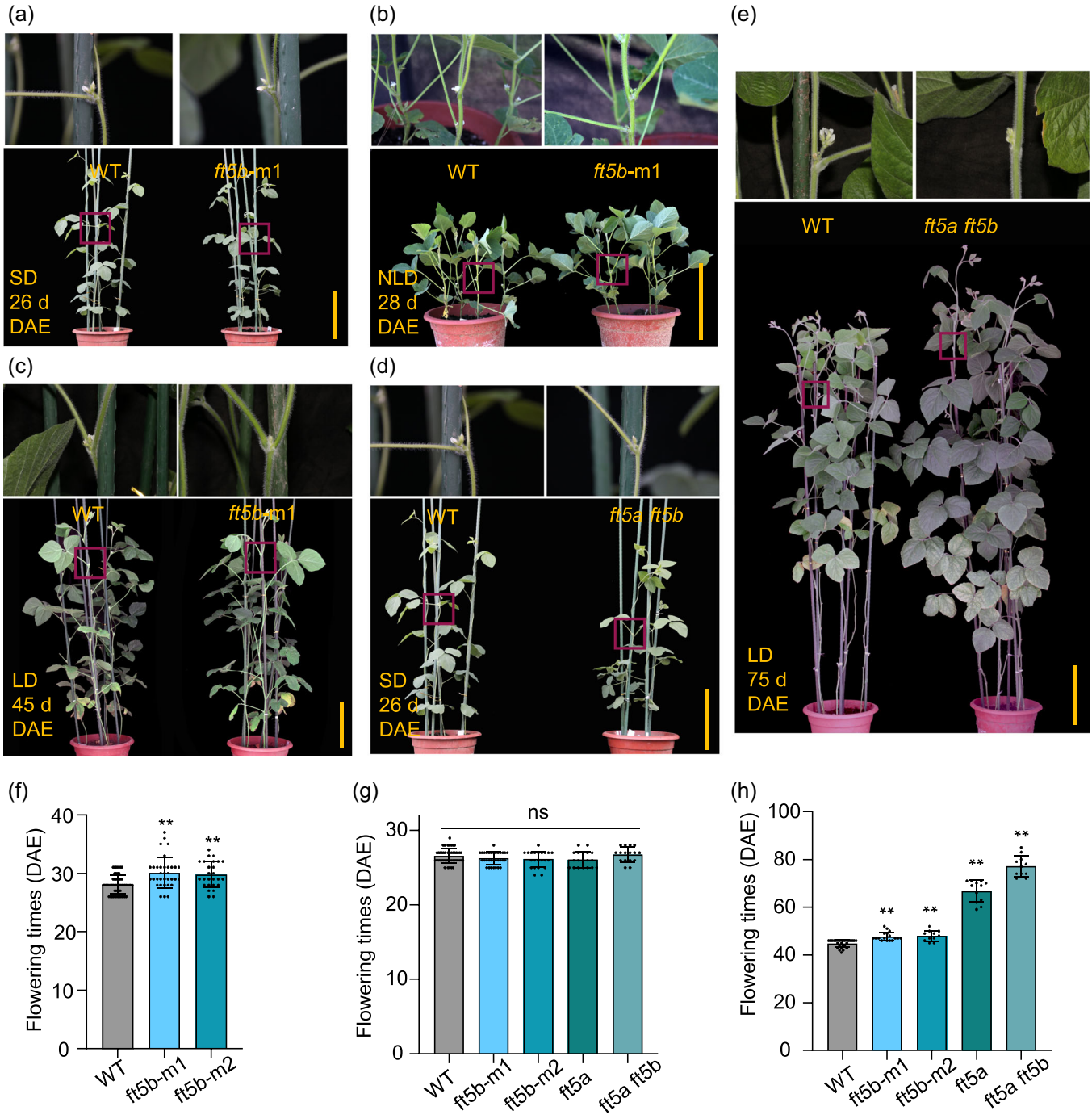

FIGURE 2 *ft5b* and *ft5a ft5b* mutant plants confer late flowering phenotypes in soybean. Phenotypes of WT plants and *ft5b* mutant plants under SD (a), NLD (b), and LD (c) conditions. Phenotypes of WT and *ft5a ft5b* mutant plants under SD (d) and LD (e) conditions. Flowering times of *ft5b* under NLD (f) conditions. Flowering times of *ft5b*, *ft5a*, and *ft5a ft5b* plants under SD (g) and LD (h). The *ft5b*/*ft5a ft5b* plants displayed significant late flowering compared with WT (**$p < 0.01$; *$p < 0.05$). Error bars represent standard deviation. Scale bar = 30 cm. DAE, days after emergence; LD, long day; NLD, natural long-day; ns, nonsignificant; SD, short-day; WT, wild type.

GmLFY2 were significantly upregulated under different photoperiod conditions (SD, NLD, and LD) (Figure 3a–c). However, there were no significant changes in the expression of *GmSOC1a* and *GmSOC1b* under any photoperiod condition in OE2 plants (Figure 3a–c).

Conversely, in *ft5b* mutants, the transcription of two *GmFUL* genes and two *GmSOC1* genes was slightly downregulated under SD conditions but remained unaffected under NLD conditions (Figure 3a,b). Notably, the expression of four *GmAP1* genes was significantly decreased under both NLD and LD conditions in *ft5b* mutants (Figure 3b,c). Additionally, the expression of two *GmFUL* genes, *GmLFY1* and *GmLFY2*, was significantly downregulated under LD conditions (Figure 3c). These findings demonstrate that

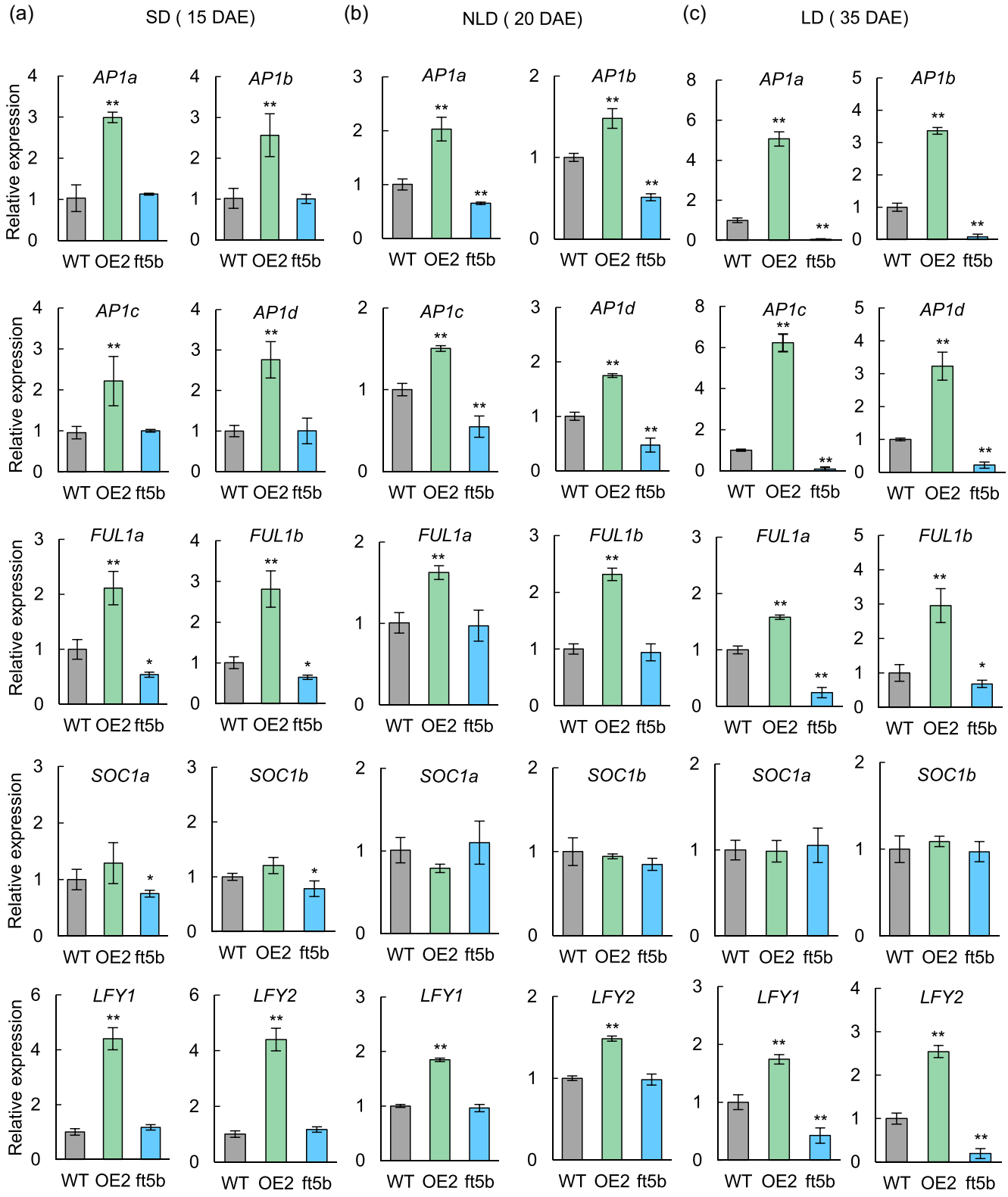

FIGURE 3 Expression patterns of flowering-related genes in WT, *ft5b* mutants, and *GmFT5b* transgenic (OE) plants under SD (a), NLD (b), and LD (c) conditions. *GmAP1a* (Glyma.01G064200), *GmAP1b* (Glyma.01G064200), *GmAP1c* (Glyma.08G269800), *GmAP1d* (Glyma.02G121600), *GmLFY2* (Glyma.06G163600), *GmFULa* (Glyma.06G205800), *GmFULb* (Glyma.04G159300), *GmSOC1a* (Glyma.18G224500) and *GmSOC1b* (Glyma.09G266200). The relative transcript levels of flowering-related genes were normalized to the internal standard *GmActin* (Glyma.18G290800). The *ft5b*-m1 and OE2 plants were used for gene expression analysis. Error bars represent standard error. * and ** represent significant differences between transgenic lines or *ft5b* mutant and WT plants at $p < 0.05$ and 0.01 (Student's *t* test). LD, long day; NLD, natural long-day; SD, short-day; WT, wild type.

GmFT5b significantly impacts the expression of established downstream flowering-related genes, which are crucial for controlling the timing of flowering in soybeans under different photoperiod conditions.

3.5 | GmFT5b protein physically interacts with the GmFDL15 protein

To unravel the underlying molecular mechanisms of *GmFT5b* in regulating flowering time, we conducted a yeast two-hybrid (Y2H) screening assay using a soybean cDNA library. Among the 10 unique putative interacting proteins identified (Supporting Information: Table S1), we focused on the bZIP transcription factor Glyma.15G222400, known as GmFDL15 (Nan et al., 2014), due to its potential involvement in FT/FD complex formation and its coexpression-with-GmFT5b-in the SAM under LD conditions (Supporting Information: Figure S4A). GmFDL15 consists of 302 amino acid (aa) residues and contains a bZIP domain (aa residues 228–296) and a SAP (RXXS/TAP) motif (aa residues 296–302), which has been reported as a putative binding sequence for FT proteins (Abe et al., 2005; Nan et al., 2014) (Supporting Information: Figure S4B). Multiple alignment analysis using DNAMAM software further confirmed the presence of a highly conserved bZIP domain (N-x7-R-x9-L-x6-L-x6-L) and SAP motif in the GmFDL15 protein sequence (Supporting Information: Figure S4C), indicating that GmFDL15 is a typical FD protein homolog.

To validate the interaction between GmFT5b and GmFDL15, we conducted several assays. Y2H experiments confirmed a strong interaction between GmFT5b and GmFDL15in yeast cells, while GmFT5a and GmFT2a did not interact with GmFDL15 (Figure 4a). Similarly, GmFT5b did not interact with GmFDL06, GmFDL08, GmFDL12, GmFDL13, GmFDL15, and GmFDL19 (Figure 4d). Further Y2H assays revealed that GmFT5b interacted with the C-terminal region of GmFDL15 (residues 201–302 aa), while GmFT5b did not interact with N-terminal regions GmFDL15-1 (residues 1–100 aa) and GmFDL15-2 (residues 101–200 aa) (Supporting Information: Figure S4E). The bimolecular fluorescence complementation (BiFC) assay further confirmed the physical interaction of GmFT5b and GmFDL15 in the nucleus of *Nicotiana benthamiana* leaves (Figure 4b). Additionally, MBP pull-down assays with heterologously expressed fusion proteins demonstrated that GST-GmFDL15 protein could interact with MBP-GmFT5b, confirming the interaction (Figure 4c). Together, these results prove that GmFT5b physically interacts with GmFDL15, indicating a potential conserved and cooperative function of these two proteins in soybeans.

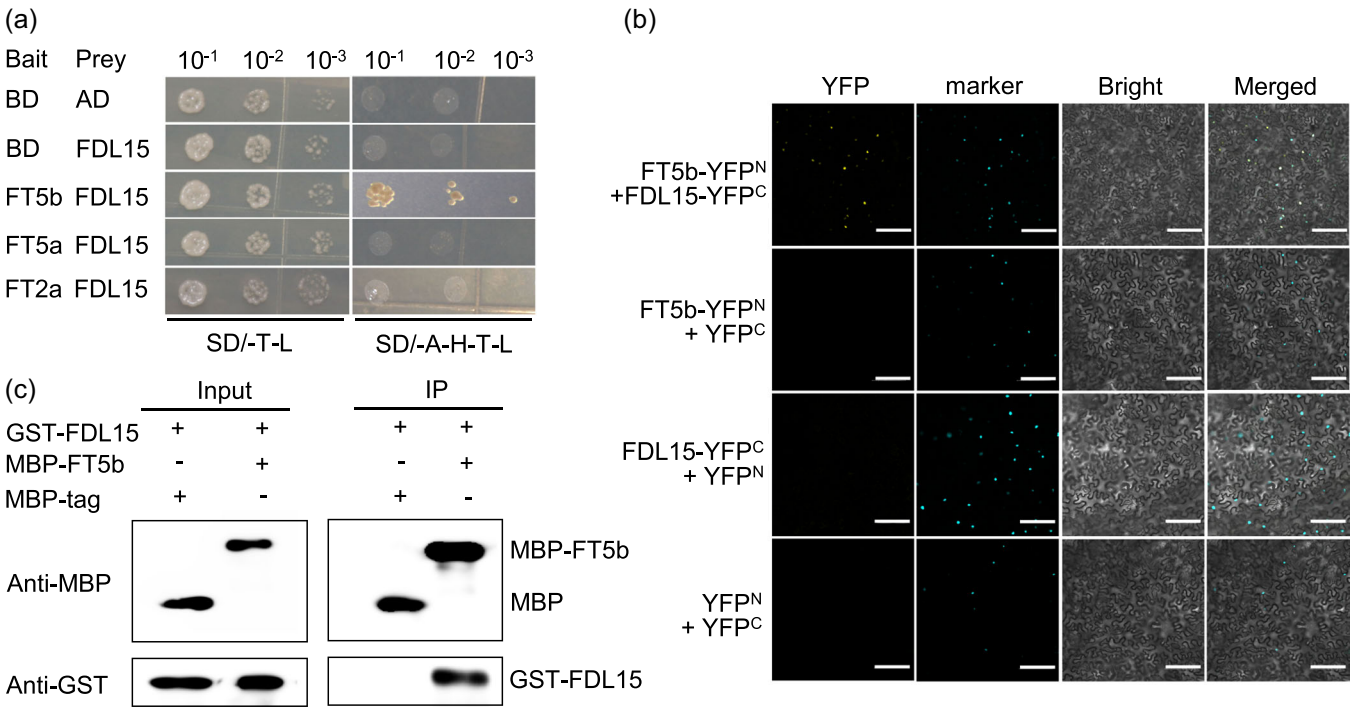

FIGURE 4 Interaction analysis of GmFT5b with GmFDL15 protein. (a) Yeast two-hybrid analysis of the interaction of GmFT5b and GmFDL15 protein. Cotransformed yeast clones were diluted and then placed on double dropout supplements (SD/-T-L) and quadruple dropout supplements (SD/-A-H-T-L) to detect reporter gene expression. FT5b represents GmFT5b-pGKBT7. AD represents pGADT7. BD represents pGKBT7. FDL15 represents GmFDL15-pGADT7. (b) BiFC analysis of GmFT5b and GmFDL15 protein interaction in *Nicotiana benthamiana* leaves. (c) GmFT5b interacts with GmFDL15 in a pull-down assay. GST-tagged GmFDL15 was incubated with MBP-agarose bound with MBP-GmFT5b or MBP proteins, and the immunoprecipitated proteins were detected with anti-GST antibodies. MBP-FT5b represents MBP-GmFT5b fusion protein. GST-FDL15 represents GST-GmFDL15 fusion protein.

3.6 | Haplotype differentiation of *GmFT5b* assists in the expansion of soybean regional adaptability

To investigate the impact of *GmFT5b* mutations on soybean adaptability, we analyzed 160 soybean accessions cultivated in China. We identified four major haplotypes (H1–H4) for the *GmFT5b* gene, with sequence polymorphisms found in both coding and noncoding regions, including a nonsynonymous mutation in the first exon (Figure 5a). Subsequently, we assessed the flowering and maturity times of these accessions at six different latitudes in China during 2017 (Figure 5b,c). Accessions with H1 showed earlier flowering and maturity times in all regions. Conversely, those with H2 generally flowered and matured later, and 24 accessions did not flower in the high-latitude region of Heihe due to the short photoperiod (Figure 5b,c). Interestingly, 71.43% of low-latitude accessions carrying H2 exhibited delayed flowering and maturity under short photoperiod conditions (Figure 5b,c). H3 and H4 showed no significant difference in their effects, but they did have a modest impact on flowering and maturity.

We then examined the geographical distribution of the *GmFT5b* haplotypes in the 160 soybean accessions. Accessions carrying H1 were predominantly found in the Huang-Huai-Hai Rivers Valley (32° N–40° N) and North China (38° N–53° N) regions (Supporting Information: Figure S5). H1 likely plays a role in shortening the growth period at high latitudes. Interestingly, accessions with H2 were widely distributed across China, and among them, 71.43% were low-latitude accessions, which might delay flowering and maturity under short photoperiod conditions (Figure 5b,c, Supporting Information: Figure S5). H3 was relatively widespread among Chinese accessions, ranging from latitudes of 21.9° N to 47.6° N (Supporting

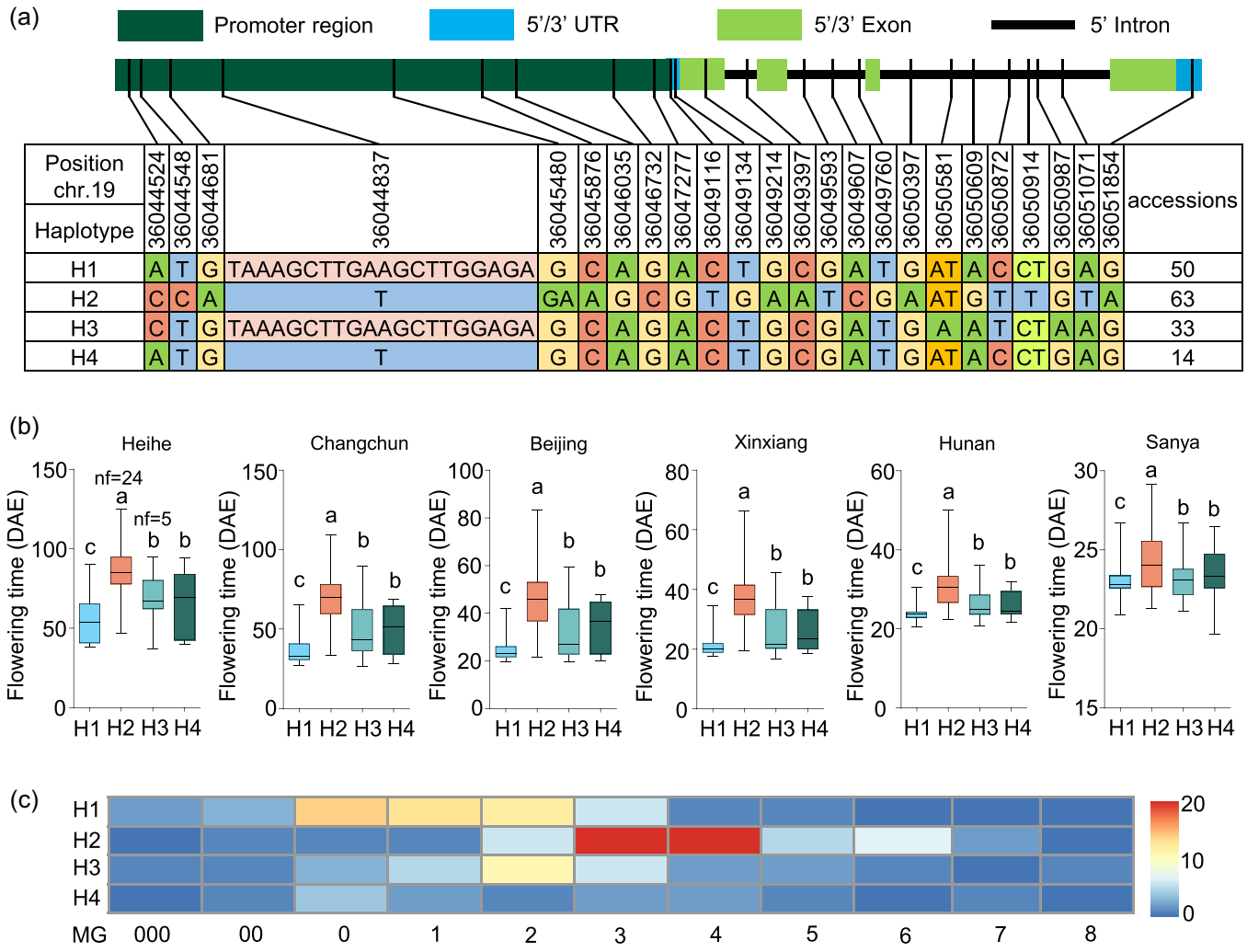

FIGURE 5 Phenotypic associations analysis of *GmFT5b* in soybean accessions. (a) Four haplotypes of *GmFT5b* in soybean accessions. The gene regions of *GmFT5b*, site numbering, and physical positions in 160 soybean accessions were compared with the reference genome sequence of Williams 82 variety, respectively. The different colours denote nucleotide mutations. (b) The flowering times of accessions carrying different *GmFT5b* haplotypes. (c) The maturity time of accessions carrying different *GmFT5b* haplotypes. The coloured bar denotes the number of soybean accessions. The maturity groups were divided into 10 groups. The field trials were carried out in Heihe, Changchun, Beijing, Xinxiang, Hunan, and Sanya, China, in 2017. nf indicates the number of varieties that did not flower after 130 DAE. Different letters represent significant differences determined by one-way ANOVA ($p < 0.05$). DAE, days after emergence; MG, maturity groups.

Information: Figure S5). Accessions carrying H4 were mainly found in the central and northeast regions of China (Supporting Information: Figure S5). Our findings indicate that natural variations in *GmFT5b* contribute to the broad adaptability of soybeans, particularly with the H1 genotype potentially shortening the flowering time at high latitudes.

3.7 | *GmFT5b* modulates flowering in defined regions under *E1* locus control

E1 is known to delay soybean flowering by directly inhibiting *FT* gene expression (Lin et al., 2022; Xia et al., 2012). Interestingly, qPCR analysis revealed that the expression of *GmFT5b* was significantly higher in *E1* RNAi soybean plants (in ZGDD with the *E1* allele) and *E1*-knockout soybean plants (in Zhonghuang42 with the *E1* allele) compared to WT plants (Figure 6a,b). This suggests that *GmFT5b* is regulated by *E1* in soybean.

Notably, the E1 haplotype significantly influences soybean adaptation by affecting flowering times (Liu et al., 2020). The e1-as haplotype, a leaky allele retaining partial function, contributes to the adaptability in regions with shorter growing seasons at higher latitudes (Lin et al., 2020; Xia et al., 2012). Moreover, E1 accessions inhibit flowering from low to high latitudes, while e1-as accessions exhibit early flowering phenotypes (Figure 6c). Interestingly, the combination of E1 and H1 haplotypes (E1H1 accessions) results in significantly earlier flowering and maturity in all tested regions, whereas the E1H2 accessions display delayed flowering and maturity, particularly in the high-latitude areas (Figure 6d,f). In contrast, the e1-asH1 (i.e., accessions carrying both e1-as and H1 haplotypes) to e1-asH4 accessions exhibit similar flowering and maturity times across the six environments (Figure 6e,g), indicating that e1-as plays a dominant role in determining early flowering in soybeans. These results underscore the significant influence of the *E1* locus in flowering control, while *GmFT5b* may fine-tune flowering in specific regions under the control of the *E1* locus.

4 | DISCUSSION

FT and its homologs are widespread in plants and primarily function in the photoperiod pathway to regulate flowering time, as demonstrated in *Arabidopsis* and rice (Corbesier et al., 2007; Kojima et al., 2002). However, the soybean genome is more complex than *Arabidopsis* and contains at least 11 *FT* genes with diverse functions, suggesting that their regulatory mechanisms in flowering are also considerably more intricate in soybean (Kong et al., 2010; Lin et al., 2020; Liu et al., 2018; Wang et al., 2015). In this study, we unveiled a molecular pathway mediated by the GmFT5b/GmFDL15 complex that induces flowering in soybeans. Natural variations in *GmFT5b* play a critical role in improving geographical adaptation, but it is noteworthy that the *GmFT5b* locus is strongly influenced by the *E1* locus, which further adds to the complexity of flowering regulation in soybeans.

In soybean, the functions of *GmFT1a*, *GmFT2a/b*, *GmFT3a/b*, and *GmFT5a* in regulating flowering and maturity have been studied (Cai et al., 2020; Chen et al., 2020; Liu et al., 2018; Su et al., 2022; Takeshima et al., 2019; Yuan et al., 2022), but the role and mechanism of *GmFT5b* remained unclear. In this study, we demonstrated that *GmFT5b* is a conserved positive regulator of floral development in soybeans. Overexpression of *GmFT5b* resulted in early flowering (Figure 1), while loss of *GmFT5b* function led to delayed flowering, specifically under noninductive LD conditions (Figure 2). Intriguingly, *FT* homologs can induce plants to flower under noninductive conditions. For instance, the upregulated *PPDH2* promotes flowering under SD photoperiod in barley and wheat, while the leakiness of rice *RFT1* expression results in flowering under LD photoperiod (Casao et al., 2011; Itoh & Izawa, 2013). However, *GmFT5a* and *GmFT5b* showed no differential expression in all photoperiods (Liu et al., 2018). *GmFT2a* primarily influenced glowering under SD conditions, potentially overcoming the effect of flowering inhibitors without the need for other *FTs* like *GmFT2b*, *GmFT5a*, and *GmFT5b* (Cai et al., 2020; Chen et al., 2020). However, under LD conditions, soybeans required additional flowering activators, such as *GmFT5a* and *GmFT5b*, to overcome and promote flowering inhibitors. We speculated that *GmFT5b*, in combination with *GmFT5a* and *GmFT2b*, synergistically promoted the transition to reproductive growth under noninductive conditions (Kong et al., 2010; Liu et al., 2018). Interestingly, the GCR in the *ft2a* or *ft5a* single mutants resulted in a weak acceleration of flowering, accompanied by elevated expression levels of other *FT* genes (Li et al., 2021). Similarly, *GmFT5a* was highly expressed in *ft5b* mutant plants (Supporting Information: Figure S3). Remarkably, the *ft5a ft5b* double mutants exhibited extremely late flowering compared to any single mutants under LD conditions (Figure 2h), suggesting that *GmFT5b* can overcome the limitation of *GmFT5a* in the double mutants. Therefore, a loss-of-function mutation in *GmFT5b* is partially compensated by *GmFT5a* in photoperiodic flowering and the phenotype-enhancing *ft5a ft5b* double mutants offer valuable resources for soybean breeding.

FD homologs are widespread among photoperiod-sensitive plant species, and soybeans have a minimum of 18 GmFDL proteins (Collani et al., 2019; Nan et al., 2014). Interestingly, GmFDL12 and GmFDL19 interact with both GmFT2a and GmFT5a, while GmFDL06 interacts exclusively with GmFT5a, suggesting potential functional divergence of *FT* genes (Takeshima et al., 2019). In our study, we observed a unique interaction between GmFT5b and GmFDL15, indicating that GmFT5b regulates flowering through a distinct pathway. In *Arabidopsis*, the FT-FD complexes induce the expression of *AP1* genes and promote flowering (Balanzà et al., 2018). Similarly, our research found that *GmFT5b* overexpression led to increased expression of *GmAP1* genes, resulting in early flowering, whereas *GmFT5b* impairment significantly reduced the abundance of *GmAP1* genes, leading to delayed flowering. This suggests that the GmFT5b-GmFDL15 complex may regulate soybean flowering by controlling the expression of *GmAP1* genes. In soybean, the *E1* gene is activated by LD conditions, leading to the repression of *GmFT2a* and *GmFT5a*

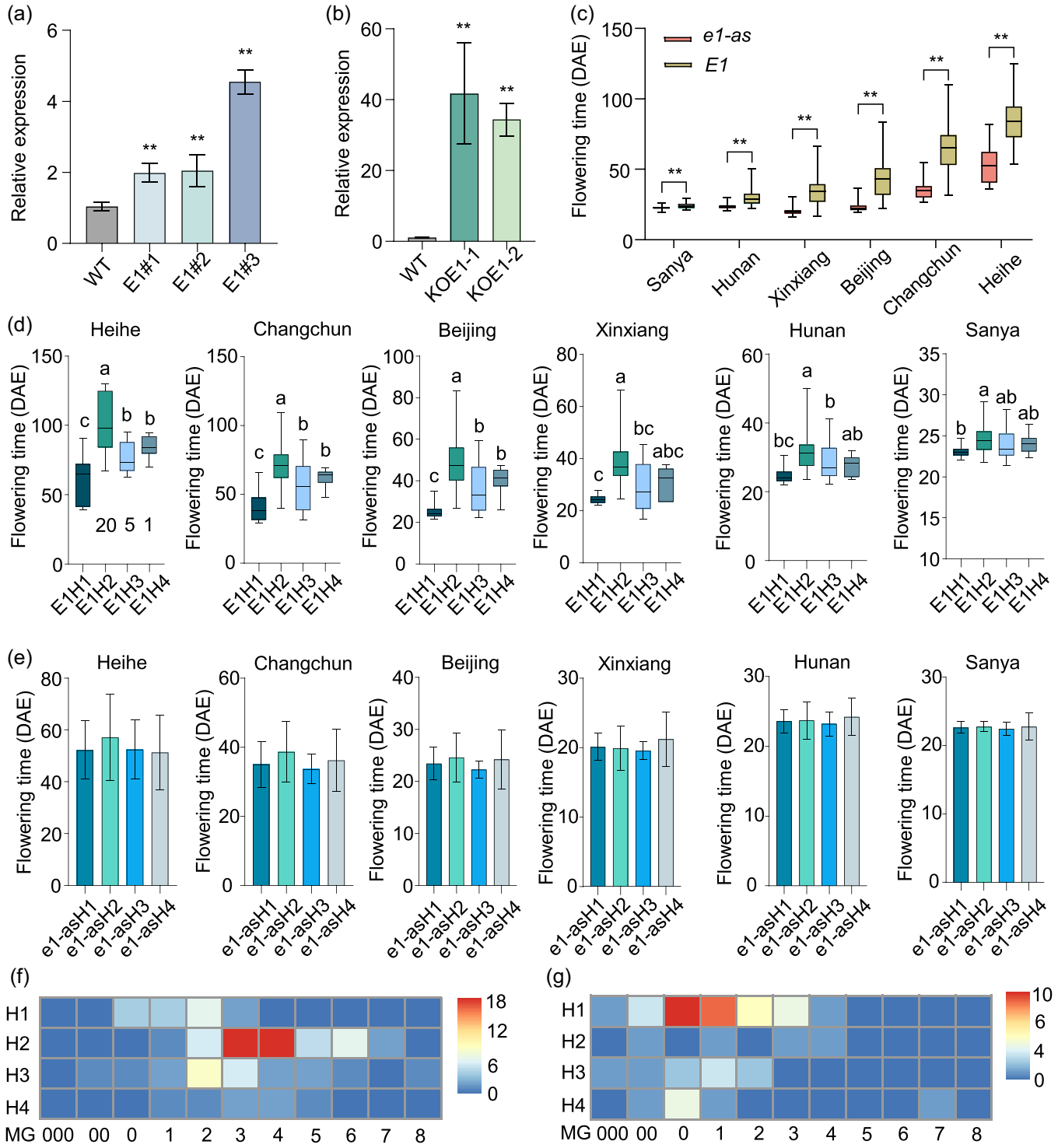

FIGURE 6 Phenotypic association analysis and the interactions of *GmFT5b* and *E1* in soybean accessions. The expression of *GmFT5b* in *E1*-RNAi (a) and *E1*-knocked out (b) plants. (c) Association mapping analysis of *E1* in soybean accessions. The flowering times of accessions carrying different *GmFT5b* haplotypes under *E1* (d) or *e1-as* (e) background. The maturity times of accessions carrying different *GmFT5b* haplotypes under *E1* (f) or *e1-as* (g) background. The coloured bar denotes the number of soybean accessions. The maturity groups were divided into 10 groups. MG, maturity groups. The field trials were carried out in Heihe, Changchun, Beijing, Xinxiang, Hunan, and Sanya, China, in 2017. DAE, days after emergence. nf indicates the number of varieties that did not flower after 130 DAE. Values are means ± SD. Different letters represent significant differences determined by one-way ANOVA ($p < 0.05$).

expression and inhibiting flowering. Conversely, under SD conditions, *E1* is inhibited, allowing *GmFT2a* and *GmFT5a* to promote flowering (Lu et al., 2017). Intriguingly, we observed that the expression levels of *GmFT5b* were significantly elevated after knocking down or knocking out *E1* (Figure 6a,b), suggesting that *E1* acts upstream of the flowering pathway and negatively regulates *GmFT5b*.

Soybeans exhibit a wide geographical distribution, but their adaptability to different latitudes is limited due to their photoperiod sensitivity (Cai et al., 2020; Chen et al., 2020; Li et al., 2021; Wang et al., 2020). In our study, we identified four *GmFT5b* haplotypes associated with flowering and maturity, which are crucial factors influencing soybean adaptability (Figure 5b,c). The *E1* locus in soybeans is known to play a central role in the photoperiodic response mechanism, directly repressing *GmFTs* and determining the diversity and distribution of soybean varieties (Lin et al., 2020; Liu et al., 2020; Xia et al., 2012). Our findings revealed that the effects of *GmFT5b* haplotypes were strongly influenced by the *E1* locus (Figure 6). Notably, *GmFT5b* haplotypes appeared to have a significant function mainly in varieties with the *E1* haplotype (Figure 6d), suggesting that *E1* may act upstream to mediate the function of *GmFT5b* in flowering. However, the specific regulatory relationship between *E1* and *GmFT5b* requires further investigation. These results underscore the epistatic nature of the *E1* locus over the effect of *GmFT5b* and imply that *GmFT5b* haplotype data could be valuable guidance for breeders in improving soybean varieties through genetic improvement.

In conclusion, our study demonstrates that GmFT5b plays a crucial role in positively regulating soybean flowering, and this function can significantly impact the geographical distribution of soybean accessions. These findings shed light on the role of *GmFT5b* in the soybean photoperiod pathway and highlight how natural variations in *GmFT5b* contribute to the geographical adaptability of soybeans to higher latitudes.

ACKNOWLEDGEMENTS

We gratefully acknowledge Weiwei Yao and Guo Li for their assistance in soybean transformation. This work was supported by Major Science and Technology Projects of China (2016ZX08010-004), the National Natural Science Foundation of China (31871644), the Ministry of Science and Technology of China (2016YFD0100504), and the CAAS (Chinese Academy of Agriculture Sciences) Agricultural Science and Technology Innovation Project.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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

How to cite this article: Su, Q., Chen, L., Cai, Y., Wang, L., Chen, Y., Zhang, J. et al. (2023) The *FLOWERING LOCUS T 5b* positively regulates photoperiodic flowering and improves the geographical adaptation of soybean. *Plant, Cell & Environment*, 1–13. https://doi.org/10.1111/pce.14739