GmFT4, a Homolog of FLOWERING LOCUS T, Is Positively Regulated by E1 and Functions as a Flowering Repressor in Soybean

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Abstract

The major maturity gene E1 has the most prominent effect on flowering time and photoperiod sensitivity of soybean, but the pathway mediated by E1 is largely unknown. Here, we found the expression of GmFT4, a homolog of FLOWERING LOCUS T, was strongly up-regulated in transgenic soybean overexpressing E1, whereas expression of flowering activators, GmFT2a and GmFT5a, was suppressed. GmFT4 expression was strongly up-regulated by long days exhibiting a diurnal rhythm, but down-regulated by short days. Notably, the basal expression level of GmFT4 was elevated when transferred to continuous light, whereas repressed when transferred to continuous dark. GmFT4 was primarily expressed in fully expanded leaves. Transcript abundance of GmFT4 was significantly correlated with that of functional E1, as well as flowering time phenotype in different cultivars. Overexpression of GmFT4 delayed the flowering time in transgenic Arabidopsis. Taken together, we propose that GmFT4 acts downstream of E1 and functions as a flowering repressor, and the balance of two antagonistic factors (GmFT4 vs GmFT2a/5a) determines the flowering time of soybean.

Introduction

The transition from vegetative to reproductive stage is a critical event in the life cycle for seed-propagated plants. Seasonal changes in day length are perceived in leaves, while the responses occur at the apex by long-distance signaling. Florigen, the molecule(s) that produced in leaves, is a florigen that moves through the phloem to the shoot apical meristem (SAM) [1–4].

The expression of FT is principally regulated by the CONSTANS (CO) gene, a central regulator that accelerates flowering in the long day pathway (for long day plants), which is modulated by the circadian clock and day length [8]. The photoperiodic response in Arabidopsis thaliana requires the precise regulation of CO and FT expression coinciding with a photosensitive phase [9–10].

Apart from FT, two other PEBP family members, TWIN SISTER OF FT (TSF) and TERMINAL FLOWER 1 (TFL1), are also involved in the control of flowering. TSF is a flowering activator, and TFL1 is a flowering repressor. TSF is the closest homolog of FT in Arabidopsis and is thought to be an additional integrator of flowering time pathways. The mRNA levels of TSF and FT showed similar patterns of diurnal oscillation and response to photoperiods [11]. Both FT and TSF are expressed in the vascular tissue of plant leaves but are spatially different, with TSF expressed mainly in hypocotyls while FT expressed in cotyledons and leaves [11,12]. TFL1, a shoot meristem identity gene, is expressed specifically in the shoot apical meristem (SAM) and represses the transition to flowering [13–16].

FT acting as a floral activator is widely conserved in plant species, although FT mRNA can be regulated by distinct mechanisms among different species even within long-day (LD) or short-day (SD) plant species [17]. Overexpression of FT orthologs, HaFTa and RFT1, generally showed an early-flowering phenotype, while mutations in FT led to a late flowering phenotype in rice [18–20]. Similarly, many functional FT orthologs were characterized, e.g. GmFT2a and GmFT5a in soybean (Glycine max) [21–22], ZC8 in maize (Zea Mays) [23–24], SFT in tomato (Solanum lycopersicum) [25], TaFT in wheat (Triticum aestivum) [26], HaFT in barley (Hordeum vulgare) [27], PhFT1/2 in Pharbitis (Pharbitis nil) [28], HaFT1 and HaFT4 in
sunflower (Helianthus annuus) [29]. In sugar beets, two FT orthologs BrFT1 and BrFT2 act antagonistically. BrFT2 is functionally conserved with FTs from other plants and is essential to activate flowering. In contrast, BrFT1 represses flowering and is crucial for the vernalization response in sugar beets [30].

It is generally accepted that the clock-controlled CO-FT external coincidence mechanism is conserved in higher plants. However, each plant species has evolved its own unique mechanisms to induce flowering under optimal conditions. In rice, Hhl1, an ortholog of CO, promotes flowering under SD conditions, while another rice CO-like gene, Ghd7, acts as a floral repressor under LD conditions and suppresses the transcription of Hhl1, a floral activator of multiple flowering signals [20,31–32]. In barley, HeCO9, an ortholog of CO, acts as a negative regulator of flowering under non-inductive SD conditions. In Pharbitis, PbFT mRNA abundance was not related to PbCO expression [28]. It was reported Lotus japonicas (a model legume) might lack the upstream positive regulator CO [33]. In pea (Pisum sativum), COIs is the most homologous gene, but is not the ortholog to ACO in terms of the function. The diurnal expression rhythm of COIs under long days is more similar to Arabidopsis CO1 and CO2 [34], which have little effect on flowering time [35], and the expression of COIs is not obviously altered in late1 mutants (LATE1 is an ortholog of Arabidopsis GIGANTEA) [34], while in Arabidopsis ACO expression is constantly dampened in gi-2 mutants [36]. In soybean, the maturity gene EI, which has the most prominent effect on flowering time and photoperiod sensitivity, is a legume-specific gene [37]. Hence, it is speculated that there might be significant differences in the mechanisms of flowering time regulation between legume and the model species Arabidopsis/rice.

"Photoperiodism " in soybean was discovered in 1920, but the molecular mechanism is poorly understood. Soybean is typically a short-day (SD) photoperiod-sensitive plant: flowering is induced when the daylength becomes shorter than a critical length. Each soybean cultivar is generally restricted to a very narrow range of latitudes due to photoperiod sensitivity. Flowering time and maturity in soybean are important quantitative traits that contribute to photoperiod adaptability, domestication, and productivity.

To date, eight flowering time or maturity loci, designated EI to E8 [38–45], along with the J locus for the long juvenile period trait, with which soybean flowers late even under short days (SDs) [46], have been characterized genetically. Of these, EI, E3, and E4 are involved in photoperiod response [40–41,44,47–49]. E3 and E4 encode homologs of the photoreceptor phytochrome A (PHYA) [50–52]. E2 encodes a homolog of GIGANTEA (GI) [53], a key regulator of photoperiodic flowering in Arabidopsis that functions upstream of CO and FT [36,54]. E1 encodes a legume-specific protein, which contains a putative bipartite nuclear localization signal, a region distantly related to DNA-binding B3 domain and a helix–turn–helix structure, and might function as a transcription factor [37]. In addition, two of the FT homologs, GmFT2a and GmFT3a, are responsible for inducing flowering under short-day conditions [22–23]. GmFT2a and GmFT3a are regulated by PHYA: Functional E3 and E4 genotypes suppressed the expression of GmFT2a and GmFT3a under long-day conditions and delayed flowering, whereas double-recessive e3e4 genotypes induced GmFT2a and GmFT3a expression and promoted early flowering regardless of day length [22–23].

In our previous study, we proposed that EI is a part of the phytochrome A signaling pathway and antagonistically determines the expression level of GmFT2a and GmFT3a [37]. Long-days (LDs) are necessary for the induction of EI expression, whereas loss-of-function alleles at E3 or E4 can result in some degree of suppression of the EI transcription and correspondingly elevated GmFT2a and GmFT3a expression. When a functional EI gene was transformed into the early-flowering cultivar Kariyutaka, transgenic plants overexpressing EI displayed late flowering and suppression of GmFT2a and GmFT3a transcript levels, indicating that the transcript level of EI was negatively correlated with that of GmFT2a and GmFT3a, but positively with flowering time.

In this study, we found the transcript level of FT ortholog GmFT4 (Glyma09g47810) in soybean was strongly up-regulated in transgenic soybean overexpressing EI, and is tightly associated with EI or ei-as expression in soybean cultivars. Ectopic expression analysis in Arabidopsis demonstrated that GmFT4 acts as a flowering repressor. The diurnal rhythm and tissue-organ expression pattern of GmFT4 were also analyzed. Taken together, we propose that GmFT4 is a key regulator in the EI-mediated photoperiodic flowering pathway, and soybean has developed its unique pathway to control flowering through coordinated regulation between the flowering promoters GmFT2a/GmFT3a and repressor GmFT4.

Materials and Methods

Plant Materials and Growth Conditions

Soybean cultivars Kariyutaka, HeiNong48, Mufeng7, HN112, HN39, HX3, EI near-isogenic line Harosoy-E1 and Harosoy-e1, Jack, Jinlin35, Sidou11, Yanhuang3 and Sakamotowase were used. Kariyutaka, HeiNong48, Mufeng7 and Sakamotowase are photoperiod-insensitive cultivars and flower early under both SDs and LDs. HN112, HN39, HX3, Jack, Jinlin35, Sidou11 and Yanhuang3 are photoperiod-sensitive cultivars and flower late under LDs. HX3 exhibits the long juvenile period trait, and flowers late even under SDs. Harosoy-E1 and Harosoy-e1 are EI near-isogenic lines. Harosoy-E1 carrying the dominant functional EI allele is a late flowering phenotype. Harosoy-e1 carrying the recessive ei allele, with a single missense point mutation, demonstrates an early flowering phenotype. Plants were grown in an artificial climate chamber under either SDs (12 h:12 h light/dark) or LDs (16:8 h light/dark) at 28°C under a light fluence of 200–300 μmol m⁻² S⁻¹. On the 16th day after emergence, fully expanded trifoliolate leaves were sampled 4 h after dawn from three individual plants for real-time PCR analysis.

For diurnal rhythmic expression analysis, soybean cultivar Harosoy-E1 was used. Soybean plants were kept under SDs (12 h of light) and LDs (18 h of light) for 16 days before being transferred into continuous light or dark conditions. Pieces of fully expanded trifoliolate leaves from three individual plants were sampled every 2 h starting at dawn under SD, LD and continuous light conditions, and sampled every 4 h under continuous dark conditions for real-time PCR analysis.

For tissue-organ analysis, soybean cultivars Kariyutaka, transgenic soybean overexpressing EI in Kariyutaka, Harosoy-E1, Harosoy-e1 and HX3 under LDs were used. Three sets of unifoliolate leaves, unexpanded and fully expanded trifoliolate leaves, apical meristems, petioles, stems and flowers from three individual plants were sampled for real-time PCR analysis.

RNA Isolation, cDNA Synthesis and Quantitative Real-time PCR Analysis

Total RNA from leaves, apical meristems, petioles and stems was extracted using TRZol (Invitrogen, Carlsbad, CA, USA) method and total RNA from flowers was extracted using TransZol plant (TransGen, Beijing, China) according to the manufacturer’s instructions. The RNA was treated with RNase-free recombinant DNase I (TaKaRa, Dalian, China). The integrity of the RNA was...
checked electrophoretically and quality assessment of total RNA was checked with NanoDrop™ ND-2000 c Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The isolated RNA was then subjected to reverse transcription using the SuperScript™ III Reverse Transcriptase kit.

Quantitative real-time PCR was performed on each cDNA sample with the SYBR Green Master Mix (TransStart Top Green qPCR SuperMix, Beijing, China) on chromo4 real-time PCR detection system (Bio-Rad, USA) according to the manufacturer’s protocol. The measured Ct values were converted to relative copy-numbers using the ΔΔCt method. Amplification of TUAS was used as an internal control to normalize all data. Primers used were TUA5 (Glyme05g29000.1)-F 5’-TGCCACCTGAAAGCTTACAGTAA-GAGG-3’ and TUA5-R 5’- ACCACCGAGAACKAA-GAAGG-3’; GmFT4-F 5’-TTGTAGCCTCCATCGAGTTC - 3’ and GmFT4-R 5’- TCCTAGGCTATTTCCACGA -3’; GmFT2a-F 5’-ATCCCGATGCACCTAGCCCA -3’ and GmFT2a-R 5’- ACACCAAACGATGAATCCCCA -3’; GmFT5a-F 5’- AGCCGGAACCCCTTCAGGGGA -3’; GmFT5a-R 5’- GGTTGAGCTCTCCTTGCCCA -3’; E1-F 5’- CACTCAATTAAGCCTTTCA -3’; E1-R 5’- TTCACTCTCCTCTTATTTTGTG -3’T;To enable statistical analysis, three fully independent biological replicates were obtained and subjected to real-time PCR run in triplicate. Raw data were standardized as described previously [55].

Sequence Alignment and Phylogenetic Analysis

Protein sequences of GmFT4 and its homologs were obtained from NCBI or Phytozone and were aligned using Clustal X2 (protein weight matrix using the Gonnet Series with a gap penalty of 0.20, and a delay-divergent cutoff of 30%), and phylogenetic analysis was performed by using MEGA4 with the UPGMA method and 100 bootstrap iterations.

Ectopic Expression of GmFT4 in Arabidopsis

The coding region sequence of GmFT4 from E1 overexpression transgenic lines was first cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA) with the primer pair (5’- CTATATCAATGGACCCCCTTGTTC-3’) and (5’-AA-GAAGGGTCTTCATCTCCTGTC-3’). GmFT4 coding region was then PCR amplified from pGEM®-T-GmFT4 vector with primers pair (5’-GGCTTAAUATGGACCCCCCTTGTTC-3’) and (5’-GGTTAAUGGCCTCTCATCTGTTCC-3’), which contained a tail of 8 nt (marked as Bold fonts) in addition to the sequence specific to the target DNA fragment. The sequence was inserted into the pCAMBIA230035Su vector with the USER™ cloning technique [56], driven by the cauliflower mosaic virus 35 S promoter, with NptII as the selectable marker. Arabidopsis Col-0 plants were transformed by the floral dip method [57]. Transformants were selected on 1/2 MS medium containing 50 mg/L kanamycin. Seeds from each T1 plant were individually collected. Selected T2 plants were propagated, and homozygous overexpression lines were confirmed by semi-quantitative RT-PCR analysis using a gene specific primer pair of 5’- ATG-GAACCCCTTTGTTCTGAG -3’ and 5’- TCATCTCCTTCTGTCACCGCA-3’.

Flowering Time Measurements of Transgenic Arabidopsis

Arabidopsis plants were grown in soil in an artificial climate chamber under long-day conditions (16:8 h light/dark) at 22°C to 24°C with 60% relative humidity. Flowering time was recorded when the floral bolt was 1 cm high, meanwhile the total number of rosette leaves were counted. About ninety plants were measured and subjected to statistical analysis.

Results

GmFT4 Expression was Up-regulated in Transgenic Soybean Overexpressing E1

In our previous study, when E1 was overexpressed in soybean cultivar Kariyutaka, expression levels of GmFT2a and GmFT5a were decreased in the transgenic soybean compared with the wild-type [37]. We further investigated expression of other FT homologs in transgenic and wild-type soybean, and found that the expression of GmFT4, a homolog of FT, was increased in transgenic soybean lines SOV#L1, SOV#L2 and SOV#L3 compared with that of SVC(transformed vector only) and wild-type (Figure 1). Since GmFT4 showed an expression pattern opposite to GmFT2a and GmFT5a, further analysis of GmFT4 was performed in order to understand the functional role of GmFT4 in controlling flowering time.

GmFT4 was Grouped within the FT-like Clade and Carries Functionally Important FT Signatures

FT/FTL1 family members that have been functionally characterized from a wide range of monocotyledonous and dicotyledonous plant species were collected and subjected to phylogenetic analysis (Figure 2A). The results indicated that GmFT4 was grouped into the FT-like clade. Sequence alignment was also conducted (Figure 2B). Tyr85(Y) in FT and the corresponding His88 (H) in TFL1 that lie at the entrance to the ligand-binding pocket have been identified to be important for the functional diversification between FT and TFL1 [58]. Also, a 14-amino-acid external loop and a 3-amino-acid triad have also been reported to be critical for FT/FTL function determination [59]. This 14-amino-acid segment and triad segment evolves very rapidly in TFL1 orthologs, but kept almost unchanged in FT orthologs. The key residue at 140 which lies in the external loop segment unambiguously distinguishes FT [Gln140 (Q)] from TFL1 homologs [Asp144(D)]. As shown in Figure 2B, the functionally determinant residues for the FT clade in the GmFT4 protein are Tyr81(Y) and Gln146(Q). When compared with that of other FT proteins, the VYN triad is relatively invariant, however, the 14-amino-acid external loop in GmFT4 protein is more variable.

Figure 1. Expression analysis of FT-like genes in transgenic soybean overexpressing E1 and WT plants under LDs. Fully expanded trifoliate leaves were sampled 4 h after dawn for semi-quantitative RT-PCR. SOV#L1, SOV#L2 and SOV#L3 were T2 transgenic plants from transgenic T0 line TG4, that has three copy exogenous E1 insertions. SOV#L4 was T2 transgenic plant from transgenic T0 line TG2, that has 7–8 copy exogenous E1 insertions [37]. SVC, transformation vector only (i.e., vector control); WT, Kariyutaka. The TUAS gene was used as a control.

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GmFT4 Expression was Generally Elevated under LDs and was Associated with Flowering Time

In order to assess whether the expression of GmFT4 is involved in the photoperiod pathway, transcript levels of GmFT4 in different soybean cultivars under both LDs and SDs were investigated (Figure 3A, B). Generally, GmFT4 was highly induced under LDs, and repressed under SDs in most soybean cultivars, especially in photoperiod-sensitive cultivars, e.g. HN112, HN89, HX3, Harosoy-E1, Harosoy-e1, Jack, Jilin 35 and Sidou 11. Whereas, in photoperiod-insensitive cultivars, such as Kariyutaka, Heinong 48, Mufeng 7 and Sakamotowase, GmFT4 expression level was very low under both SDs and LDs. Relatively higher GmFT4 expression levels under SDs than under LDs were observed in Kariyutaka and Sakamotowase, however, it may not be meaningful to compare them since both were at very low levels. E1, E3, and E4 were reported to be involved in photoperiod responses. Kariyutaka has the e3e4 genotype (double recessive E3, E4) and showed a suppressed E1 expression. Both Heinong 48 and Mufeng 7 carry the e3 genotype (E3 recessive). Sakamotowase carries the e1-fs genotype (a frame shift mutation of E1). Therefore, we might be able to hypothesize E3, E4 and E1 regulate the photoperiod response of soybean via GmFT4.

Meanwhile, we found GmFT4 expression was significantly correlated with flowering time. Late flowering soybean cultivars were displaying high levels of GmFT4 expression, while early flowering cultivars showed opposite trends (Figure 3B). Even under SDs, long juvenile cultivar HX3 that exhibited delayed flowering under SDs also showed a relatively high GmFT4 expression. We then conducted correlation analysis (Figure 3C, D), where GmFT4 expression was significantly correlated with flowering time under both LDs (Figure 3C) ($r^2 = 0.9461^{***}$, n = 12, P<0.0001) and SDs (Figure 3D) ($r^2 = 0.8486^{**}$, n = 12, P<0.001), suggesting that GmFT4 might act as flowering repressor in soybean.

GmFT4 Expression is Associated with E1 Expression and E1 Genotype

To evaluate the functional consequence between GmFT4 and E1, E1 expression and E1 allelic variations were also investigated. As shown in Table 1, cultivars Kariyutaka, Heinong 48, Mufeng 7, HN112, HN89, HX3 and Harosoy-E1 carry the E1 genotype. Cultivars Harosoy-e1, Jack, Jilin 35 and Sidou 11 carry the e1-as genotype. Allele e1-as that harbors a 1-bp mutation, is a leaky allele and may retain partial E1 function. The e1-fs allele in cultivar Sakamotowase, has a 1-bp deletion, resulting in a premature stop codon, and is nonfunctional [37]. GmFT4 transcript level in Harosoy-E1 was higher than that in Harosoy-e1 under LDs (Table 1). Among cultivars carrying the E1 allele, expression level of GmFT4 fluctuated with the E1 expression level (Table 1). Apparently, higher GmFT4 expression level occurred in plants or cultivars with high expression level of E1, while low expression level of GmFT4 was coupled with lower E1 expression. Similar trends were also observed in cultivars carrying e1-as allele, however, much higher e1-as expression compared to E1 expression was associated with the equivalent amount of GmFT4 transcripts, possible due to the dosage effect since e1-as is less functional compared to E1. Statistical analysis showed that GmFT4 expression was significantly correlated with the E1 expression in cultivars carrying the E1 allele ($r^2 = 0.8756^{***}$, n = 14, P<0.0001) and in cultivars carrying the e1-as allele ($r^2 = 0.7776^{*}$, n = 8, P<0.01) (Figure 3E and F). In cultivar Sakamotowase with the e1-fs genotype, the expression of GmFT4 was at very low level. These results indicated GmFT4 expression level is dependent on the amount of functional E1 transcripts.
Figure 3. Expression analysis of GmFT4 in different soybean cultivars under SDs and LDs by real-time RT-PCR. (A) Number of days to flowering. (B) Evaluation of GmFT4 transcript levels in fully expanded trifoliolate leaves by real-time RT-PCR. Transcript levels relative to TUA5 were
Expression of GmFT4 Exhibits a Diurnal Rhythm under LDs

The diurnal rhythm of GmFT4 gene expression was analyzed by real-time PCR in trifoliate leaves sampled from cultivar Harosoy-1E. In plants under LDs, the expression level of GmFT4 exhibits a diurnal rhythm. Briefly, the expression level increased beginning at dawn, reached a maximum 4 h later, then began to decrease and reached its minimum at the end of the light phase. In the dark phase, expression level of GmFT4 began to increase again until 4 h after dawn in the next light/dark circle (Figure 4A).

When plants grown under LDs were transferred to subsequent 48 h continuous light (LD-DD) condition, the oscillation waveform was similar to that under LDs, but expression peak of GmFT4 appeared 4 h later than that under LDs in the first subjective cycle, and the expression peak drifted later by 4 h further in the second subjective cycle of darkness (Figure 4A). When plants grown under LDs were transferred to subsequent 48 h continuous light (LD-LL) condition, the expression waveform of GmFT4 kept a similar pattern during the first subjective cycle of continuous light, but there was a sharp increase at the end of the first subjective cycle (Figure 4A). During the second subjective cycle of continuous light, basal expression level of GmFT4 was generally elevated, but the rhythm became somewhat irregular.

In plants exposed to SDs, expression of GmFT4 was very low and irregular (Figure S1). However, transcript level of GmFT4 was increased after the shift to continuous light (SD-DD) (Figure 4B). The expression of GmFT4 kept rising during the first 24 h of continuous light, and there was a large and sharp increase at the end of subjective cycle (Figure 4B). During the next subjective cycle of continuous light, the high expression level of GmFT4 was maintained for a period, and then similarly there was a sharp increase at the end. By contrast, expression of GmFT4 was kept at a very low level under continuous dark following SDs (SD-DD) (Figure 4B) and showed no circadian rhythm (Figure S1). The results indicated that the maintenance of expression rhythm of GmFT4 needs a light/dark cycle, and a light phase can elevate the basal expression level.

GmFT4 Expressed Primarily in Leaves

We analyzed transcription profiles of GmFT4 in various tissues in near-isogenic lines Harosoy-1E and Harosoy-e1, cultivars Kariyutaka and HX3 (carrying J locus) under LDs by real-time PCR (Figure 5). The expression of E1 was generally tissue-specific, with high levels in mature leaves (fully expanded unifoliolate leaves and trifoliate leaves), relatively low expression levels in flowers, and very low but nearly identical levels in apical meristems, petioles and stems (Figure 5A). The expression levels of GmFT4 in fully expanded unifoliolate leaves, trifoliate leaves and flowers in the late flowering cultivar HX3 were higher than that in the corresponding tissues of the early flowering cultivar Kariyutaka. Also higher expression level of GmFT4 in fully expanded unifoliolate leaves, fully expanded trifoliate leaves and flowers was observed in Harosoy-1E than that in Harosoy-e1.

We further concurrently analyzed the expression level of E1 and GmFT4 in various tissues in E1 overexpression transgenic soybean plants and wild type (Kariyutaka). As shown in Figure 5B,
although an extremely high expression level of $E1$ was observed in all tissues analyzed in transgenic soybean overexpressing $E1$, $GmFT4$ was only highly induced in leaves (including fully expanded unifoliolate leaves and trifoliolate leaves) and slightly induced in flowers of the transgenic soybean (Figure 5C), implying that $GmFT4$ may function primarily in leaves, and the induction of $GmFT4$ by $E1$ is conditioned by other genetic or molecular factors that are primarily present in leaves.

Ectopic Expression of $GmFT4$ in Arabidopsis Delayed Flowering

In order to further understand the function of $GmFT4$ in flowering regulation, we conducted an ectopic overexpression experiment in Arabidopsis ecotype Columbia (Col-0). T$_3$ homozygous lines were obtained and were confirmed by semi-quantitative RT-PCR with gene-specific primers. As shown in Figure 6A, four transgenic Arabidopsis lines all exhibited high expression levels of $GmFT4$, and no expression was detected in wild-type or vc (transformation vector only) transgenic plants.

Transgenic Arabidopsis with overexpression of $GmFT4$ flowered later than the wild-type and vc plants under LDs (Figure 6B).
Wild-type Arabidopsis and vc plants needed only 27–29 days to flower on average, while GmFT4 overexpression lines needed 36–37 days to flower under LDs (Figure 6C). When flowering, wild-type Arabidopsis and vc plants had 11–12 rosette leaves on average, while GmFT4 overexpression lines had 14–15 rosette leaves on average (Figure 6D). Obviously, the phenotype of transgenic Arabidopsis overexpressing GmFT4 was distinct from that of other soybean flowering promoting FT homologs, GmFT2a/5a [21].

Discussion

Each soybean cultivar is agronomically adapted to a narrow range of latitude for their maximal yield due to photoperiod sensitivity, thus limiting widely distribution of elite soybean cultivars. Among the major maturity genes or QTLs that have been reported so far, the E1 gene has the most prominent effect on flowering time and photoperiod sensitivity in soybean [48,60–62], suggesting E1 is a key regulator of flowering in soybean. Phylogenetic analysis on protein sequence indicated E1 is legume-specific [37], implying that the major photoperiodic pathway in soybean might be different from that in Arabidopsis and rice. Negative correlation between E1 and GmFT2a/GmFT5a expression was observed in our previous study [37]. Here, we found that a FT ortholog, GmFT4, was positively associated with E1 in E1 overexpressing transgenic soybean (Figure 1), E1 NILs and different soybean cultivars (Figure 3B). Interestingly, unlike most FT-like genes, GmFT4 was characterized as a flowering repressor (Figure 6). These results indicated soybean has evolved a different strategy to regulate flowering time, and the E1-GmFT4 pathway is valuable for understanding the molecular mechanisms of flowering time regulation in soybean.

GmFT4 transcript level was strongly induced in E1 overexpressing transgenic soybean lines (Figure 1), and Harosoy-E1 showed a greater GmFT4 transcriptional abundance than Harosoy-e1 (Figure 3B). Allelic variations and transcriptional abundances of E1 prominently influence GmFT4 expression (Table 1). In addition, the expression pattern of GmFT4 under SDs and LDs...
was similar to that of E1 with a strong suppression under SDs and strong elevated expression under LDs (Figure 3B). GmFT4 and E1 have similar functions where both act as flowering repressors (Figure 6). All the results in this study indicate that GmFT4 is regulated by E1, and GmFT4 might be involved in the E1 mediated flowering control pathway. However, GmFT4 might be not the direct target of E1, since E1 acts as a transcriptional repressor (data not shown).

In plants, the regulation of FTs in different species is highly diversified. Most FT-like genes are flowering activators and their induction occurs only in daylength that induce flowering. Not surprisingly, high GmFT4 expression level in non-inductive LDs is consistent with its flowering repressing function. In Arabidopsis and rice, response to photoperiod is mediated by transcriptional regulation of FTs through an intersection between clock regulation and daylength. The expression of FT requires the activation of the clock output gene CO in the presence of light in Arabidopsis [10]. FT transcription is activated by CO under LDs but not under SDs, because under LDs, CO mRNA expression coincides with exposure of plants to light leading to stabilization of CO protein [9–10]. Here, we found that induction of GmFT4 occurred in non-inductive daylength, and the diurnal oscillation expression pattern could be retained to some extent when transferred to subsequent 24 h continuous light or continuous dark, indicating that GmFT4 was partly impacted by the circadian clock, and moreover that the induction of GmFT4 required the exposure of plants to light, which indicated, just like other FT-like genes, transcriptional regulation of GmFT4 also through an intersection between clock regulation and daylength.

Although GmFT4 is preferably induced under LDs while GmFT2a/5a is preferably induced under SDs, the oscillation waveforms of GmFT4 and GmFT2a/5a under respectively inductive conditions were similar, with an increase at the beginning of dawn, a peak 4 h later, and a minimum toward dusk, and then followed by an increase again, suggesting that these genes might be regulated by a similar mechanism in relation to the circadian clock.

In plants, FTs are highly conserved in different species. Arabidopsis has been used for functional confirmation of genes cloned, especially for FT homologs from different species. ZCN8 encodes a FT-related protein in a SD plant maize. Ectopic expression of ZCN8 accelerated flowering in transgenic Arabidopsis [23]. An antagonistic pair of FT homologs, BvFT1 and BvFT2 controls flowering time in LD plant sugar beet. Transgenic expression of BvFT2 in both Arabidopsis and sugar beet strongly promoted flowering, while transgenic expression of BvFT1 strongly repressed flowering in both Arabidopsis and sugar beet [29]. Previous studies indicated that the functions of FT genes are conserved between Arabidopsis and soybean. Ectopic overexpression of GmFT2a and GmFT3a in Arabidopsis showed a flowering promoting phenotype [21–22]. Conversely, when Arabidopsis FT was transformed into soybean, transgenic soybean flowered earlier [63]. Accordingly, we used Arabidopsis to confirm the function of GmFT4. When GmFT4 was overexpressed in Arabidopsis, transgenic Arabidopsis showed a delayed flowering phenotype (Figure 6).

GmFT4 was grouped within the FT-like clade and carries functionally important FT signatures, but acts as a flowering repressor. Upon detailed analysis by referring to the previous publications, we predicted that the residue in position 143 that lies in the external loop may be critical for function diversification. Most FT-like proteins carry Gly(G) or Glu(E) residue in this position, except for flowering repressors GmFT4 and BvFT1 from Beta vulgaris. However, further evidence at molecular level is needed to verify this hypothesis.

Most soybean cultivars have a short-day requirement for floral induction, so under long days, flowering and maturing are delayed and differ greatly among different cultivars. Here, we found the transcriptional abundance of GmFT4 is significantly correlated with the flowering time of different soybean cultivars under LDs, indicating that GmFT4 might be related to the flowering time regulation under LDs. Soybean cultivars grown at high latitudes are often photoperiod insensitive, because soybean plants planted in spring are required to flower under LDs during early summer and complete seed production in the limited frost-free season. Photoperiod insensitive cultivars grown at high latitude, such as Kariyutaka, Heinong 48, MuFeng 7 and Sakamotowase displayed low levels of GmFT4 expression. Even under SDs, transcriptional abundance of GmFT4 was also significantly correlated with the flowering time of different soybean cultivars. At low latitudes, soybean cultivars with the classic response to photoperiod flower early resulting in short plants and low grain yield [64], so soybean cultivars bred at low latitudes often needs the long juvenile period trait that featured as delayed flowering under SDs [65–67]. In this study, cultivar HX3, known for the Brazilian long juvenile period trait exhibited a delayed flowering phenotype under SDs. Generally, cultivars with the long juvenile period trait need a SD regime of 8 h/16 h (light/dark) to promote flowering. Here, we found cultivar HX3 also showed a relatively high GmFT4 expression even under SDs, indicating that GmFT4 might be an important determinant for flowering time regardless of day-length conditions. We assume that GmFT4 might contribute greatly to the wide adaptability of soybean to wide range of latitudes. Hence, we proposed a model for the flowering time regulation in soybean (Figure 7). In this model, all flowering promoters GmFT2a and GmFT5a, and repressor GmFT4, function downstream of E1, and the balance between the antagonistic FTs (GmFT4 vs GmFT2a/ GmFT5a ) determines soybean flowering time. Under SDs or in
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Author Contributions

Conceived and designed the experiments: HZ, ZJX. Performed the experiments: HZ, SXI, SL, HYW, XZZ. Analyzed the data: HZ, ZJX. Contributed reagents/materials/analysis tools: BHL, FJK, XHY, JL. Wrote the paper: HZ.

Supporting Information

Figure S1 Diurnal expression pattern of GmFT4 in plants grown in SDs followed by continuous dark. 2 h after beginning of the light phase under SD were used as control. Values represent means of three biological replicates; error bars indicate standard deviation. Fully expanded trifoliolate leaves from Harosoy-E1 were sampled every 2 h under short days, and every 4 h under continuous dark.

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