Characterization and Activity Analyses of the FLOWERING LOCUS T Promoter in Gossypium Hirsutum

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Abstract: Flowering transition is a crucial developmental process in cotton (Gossypium hirsutum L.), and the flowering time is closely correlated with the timing of FLOWERING LOCUS T (FT) expression. However, the mechanism underlying the coordination of various cis-regulatory elements in the FT promoter of cotton has not been determined. In this study, a 5.9-kb promoter of FT was identified from cotton. A bioinformatics analysis showed that multiple insertion–deletion sites existed in the 5.9-kb promoter. Different expression levels of a reporter gene, and the induction by sequential deletions in GhFT promoter, demonstrated that 1.8-kb of the GhFT promoter was stronger than 4.2-, 4.8-, and 5.9-kb promoter fragments. The binding sites of the CONSTANS (CO) and Nuclear Factor Y transcription factors were located within the 1.0-kb sequence upstream of the FT transcription start site. A large number of repeat segments were identified in proximal promoter regions (−1.1 to −1.4 kb). A complementation analysis of deletion constructs between 1.0 and 1.8 kb of G. hirsutum, Gossypium arboretum, and Gossypium raimondii FT promoters revealed that the 1.0-kb fragment significantly rescued the late-flowering phenotype of the Arabidopsis FT loss-of-function mutant ft-10, whereas the 1.8-kb promoter only slightly rescued the late-flowering phenotype. Furthermore, the conserved CORE motif in the cotton FT promoter is an atypical TGTG(N2-3)ATG, but the number of arbitrary bases between TGTG and ATG is uncertain. Thus, the proximal FT promoter region might play an important role affecting the activity levels of FT promoters in cotton flowering.

Keywords: cotton; FLOWERING LOCUS T; promoter analysis; flowering transition; CORE

1. Introduction

Flowering in plants is an important physiological process in the switch from vegetative to reproductive growth, and this process is strictly controlled by a complex regulatory network consisting of environmental cues and plant developmental signals [1]. The regulation of the FLOWERING LOCUS T (FT) gene in Arabidopsis thaliana is understood to involve at least six major pathways controlling
flowering: the photoperiod, vernalization, ambient temperature, age, autonomous, and gibberellin pathways. They converge at the integrator FT gene [2,3]. FT protein is produced in the vascular tissues of leaves and is translocated to the shoot apical meristem, where it can interact with the basic leucine zipper transcription factor FD to form a complex and then activate the expression of downstream genes involved in floral morphogenesis, such as APETALA1 and LEAFY [4–8].

The transcriptional activation of FT is directly mediated by CONSTANS (CO) under long-day (LD) inductive conditions [1]. CORE1 and CORE2 are the two CO responsive elements in the FT promoter that share the consensus sequence TGTG(N2-3)ATG [9]. The CONSTANS, CONSTANS-like, and TOC1 domain, which is a conserved motif at the C-terminus of CO, can interact with nuclear factor Y (NF-Y) transcription factors [9–11]. Cao et al. (2014) proposed a photoperiodic flowering recruitment model: NF-Y complexes bind to CCAAT distal to the FT promoter and recruit and stabilize the binding of CO to CORE1 and CORE2 proximal to the FT promoter to facilitate the transcriptional activation of FT [12]. Upland cotton (Gossypium hirsutum L.) is an allotetraploid species, which was formed by hybridization of the A-genome ancestor Gossypium arboretum (AA) with D-genome ancestor Gossypium raimondii (DD), followed by chromosomal doubling [13]. Homeologous gene pairs are included in the A and D subgenomes. Our group previously identified 42 CO-like (COL) homologues in the G. hirsutum genome, and a phylogenetic analysis classified them into three groups [14]. Group I contains 14 GhCOLs, which are clustered with AtCO and rice (Oryza sativa) Hd1 [15]. One homeologous pair, GhCOL1-A and GhCOL1-D, in Group I have the greatest sequence similarities with AtCO and Hd1. Furthermore, the overexpression of GhCOL1 can fully rescue the late-flowering phenotype of the co-2 mutant, suggesting that the GhCOL1 homologues act as flowering inducers in G. hirsutum [14].

To clarify the complex relationships between activation and suppression signals that regulate flowering through FT, Adrian et al. (2010) studied the conserved sequence of the FT promoter in Arabidopsis in detail. First, the 7.0-kb FT promoter sequences of Columbia (Col), Landsberg erecta (Ler), Arabidopsis lyrata, Brassica rapa, and Arabis alpine were compared. Three conserved sequence regions were located upstream, in the middle and downstream of the 7.0-kb FT promoter and are known as Blocks A, B, and C, respectively. Further investigations of the FT promoter (1.0-, 4.0-, 5.7-, and 8.1-kb segments) showed that Block A, located at the proximal end, and Block C, located at the distal end at approximately 5.0-kb, played important roles in the activity of the FT promoter mediated by CO. Furthermore, the overexpression of CO resulted in changes in the chromatin state, such as decreased LIKE HETEROCHROMATIN PROTEIN1 (LHP1) binding and increased H3K9K14 acetylation. These changes could be the results of the upregulation of FT expression, rather than a prerequisite for FT activation [16]. Furthermore, a number of other studies on the interactions between cis-acting elements on the FT promoter and corresponding transcription factors have also been conducted in Arabidopsis [17–20].

The function of the FT promoter has been studied in other plants, such as FT2a in soybean (Glycine max) [21], Hd3a in rice [22], and FT in wheat (Triticum aestivum) [23]. Our group identified a cotton FT homologous gene, GhFT, from G. hirsutum and demonstrated that it was predominantly expressed in stamens and sepals and had a relatively higher expression level during the initiation stage of fiber development. The ectopic overexpression of GhFT promoted flowering, lateral shoot growth, and leaf morphology, indicating that cotton FT regulates shoot architecture by advancing determinate growth [24,25]. Similar results have been reported by other groups [26–28]. However, details of the FT promoter in cotton, such as its activity, function, and sequence, remain unknown and require further study. Here, we analyzed the activity of the 5.9-kb promoter of GhFT using a series of deletion constructs and found that the GhFT promoter had an activity length of approximately 1.8-kb upstream of the transcription start site. Further research is warranted to examine the 1.8-kb promoter in G. hirsutum, G. arboretum, and G. raimondii; therefore, we have constructed a truncated 1.0-kb promoter, and the GUS activity induced by which was stronger and was more efficient in rescuing the late-flowering phenotype of ft-10 than the 1.8-kb promoter.
2. Results

2.1. Bioinformatics Analysis of the FT Promoters in Cotton

To identify candidate sequences for regulatory motifs, the sequences from 5.9-kb upstream of FT in G. hirsutum, G. arboreum, and G. raimondii were analyzed. The pairwise alignment revealed five Indel sites among them (Figure 1). Indels E, D, and C are located approximately 4.9-, 4.2-, and 1.6-kb upstream of the FT start codon, respectively, and do not exist in G. raimondii (Figure 1A–C). Indel B is located approximately 0.9-kb upstream of the FT initiation codon and does not exist in G. arboreum or the A subgenome promoters of G. hirsutum. Furthermore, repeat sequences (RSs) are identified approximately 1.0-kb and 1.2-kb upstream of ATG, but G. arboreum and the A subgenomes of G. hirsutum lack these RSs (Figure 1D). Indel A, closest to the FT start codon, have been deleted in the D subgenome promoter of G. hirsutum (Figure 1E).

**Figure 1.** Multiple sequence alignment of the FT promoter among G. hirsutum, G. raimondii and G. arboreum. (A) Sequence alignment of the distal FT promoter (−4900 to −5120 bp). One Indel sequence was identified and called Indel E (−4930 to −5120 bp). (B) Sequence alignment of the FT promoter (−4190 to −4305 bp). One Indel sequence was identified and named Indel D (−4260 to −4270 bp). (C) Sequence alignment of the FT promoter (−1560 to −1670 bp). One Indel sequence was identified and named Indel C (−1640 to −1670 bp). (D) Sequence alignment of the FT promoter (−4190 to −4305 bp). One Indel sequence was identified and named Indel D (−4260 to −4270 bp). (E) Sequence alignment of a proximal FT promoter region (−1 to −185 bp). One Indel sequence was identified and named Indel A (−879 to −890 bp).

2.2. Histochemical Activity Analysis of GhFT Promoter

To assess the activity of the GhFT promoter, GhFT promoter deletion constructs (1.0-, 1.5-, 1.8-, 4.2-, 4.8-, and 5.9-kb FT promoter fragments) were independently fused with GUS (Figure 2A). These
constructs were stably transformed into A. thaliana Col-0 plants using Agrobacterium-mediated genetic transformation. Histochemical GUS staining was performed in various tissues, such as seedlings, cotyledons, leaves, flowers, and siliques (Figure 2B). In seedlings and cotyledons, the GUS activities were induced by all the promoter fragments, and the 1.0-, 1.5-, and 1.8-kb fragments’ activities were higher than those of the 4.2-, 4.8-, and 5.9-kb fragments. The GUS activities induced by the 1.0-, 1.5-, and 1.8-kb fragments were higher in leaves, flowers, and siliques, in which did not induced by the 4.2-, 4.8-, and 5.9-kb fragments. We speculate that the 4.2-, 4.8-, and 5.9-kb promoter fragments may have the ability to induce transcription, but there are some upstream repressing sequences between 1.8- and 5.9-kb, which needs to be confirmed in the future. These results indicated that the activity regulator of the GhFT promoter in G. hirsutum was approximately 1.8-kb upstream of the FT start codon, but it did not exclude the effects of other distal sequences on GhFT expression.

Figure 2. Histochemical activity analyses of the GhFT promoter. (A) Alignment of the same promoter region of the 5.9-kb FT promoter from G. hirsutum, G. arboretum, and G. raimondii using mVISTA. Graphical shows base-pair identity in a range of 50–100%. Light-gray areas showed different Indels. Promoter constructs used for analyses are depicted as black boxes. A transient expression assay was performed using a GUS gene under the control of FT promoter fragments of 1.0-, 1.5-, 1.8-, 4.2-, 4.8-, and 5.9-kb in length. (B) GUS activity analysis of the GhFT promoter in transgenic plants of different deletions and different tissues. S, C, L, F, and Si represent seedlings, cotyledons, leaves, flowers, and siliques, respectively.
2.3. Sequence Analyses of the 1.8-kb FT Promoters in G. hirsutum, G. arboreum, and G. raimondii

To analyze the 1.8-kb promoter of FT, we first performed a phylogenetic analysis of G. hirsutum, G. arboreum, and G. raimondii. The constructed phylogenetic tree indicated that the 1.8-kb cotton FT promoters could be clearly divided into two types of the A and D subgenomes on the basis of the source of the promoter genome (Figure 3).

We further amplified the approximately 1.8-kb GhFT promoter sequences from the A and D subgenomes of G. hirsutum. Sequencing confirmed that the length of the GhFT-A promoter was 1771 bp, and the length of the GhFT-D promoter was 1701 bp. A bioinformatics analysis by the PlantCare program revealed that these 1.8-kb fragments contain a number of putative plant cis-elements. The predicted core promoter elements, such as the TATA-box and CAAT-box, were identified (Tables S1 and S2). In addition, some hormone-related elements were also recognized, including the TCA-element, ABRE, the TATC-box and the P-box. Light-responsive elements, such as the as-2-box, the G-box, the ATCT-motif, ACE, Box1, Box4, Pc-CMA2c, the AT1-motif, the GT1-motif, Sp1 and the TCT-motif, were also observed. There were also abiotic stress-tolerance factors, such as HSE, TC-rich repeats, MBS, and the GC-motif. Among tissue-expression motifs, the CAT-box motif and circadian rhythm-related elements also existed in these two sequences (Tables S1 and S2).

2.4. The 1.0-kb Sequence Upstream of the FT Translation Initiation Site Concentrated CORE and CCAAT Domains

Two CO-responsive elements (CORE1 and CORE2) in the AtFT promoter share a consensus sequence, TGTG(N2-3)ATG [9]. CO proteins can interact with NF-Y transcription factors [10,11]. NF-Y proteins consist of three unique subunits, NF-YA, NF-YB, and NF-YC, which form a heterotrimeric complex that binds DNA and can recognize CCAAT cis-elements [29–31]. Because our experiment showed that the strong GUS activities were induced by the 1.8-kb cotton GhFT promoter fragment (Figure 2), we next analyzed the CORE and CCAAT domains in this 1.8-kb sequence to predict the CO and NF-Y transcription factor binding sites. The CORE and CCAAT domains were concentrated in the 1.0-kb region upstream of the translation start site, but there was no CCAAT domain between 1.0 and 1.8 kb in the promoter (Figure S1A,B), which was different from in Arabidopsis [12]. However, compared with the consensus sequence TGTG(N2-3)ATG reported previously, the conserved motif of CORE in the cotton FT promoter is the atypical TGTG(N2-3)ATG, and the number of arbitrary bases between TGTG and ATG is uncertain, being one or six (Figure S1A,B). The FT/CO regulon model is highly conserved in photosensitive plants [32–34]. Thus, whether the FT/CO regulatory model is conserved in upland cotton remains to be determined.

The RSs within the 1.8-kb region upstream of the FT start codon in G. arboreum, G. raimondii, and G. hirsutum were further analyzed. We found that a large number of repeated fragments exist between 1.1- and 1.4-kb upstream of the FT promoters, where the cis-acting elements are also enriched (Figure 4).
Thus, these repetitive sequences between 1.1- and 1.4-kb upstream of the translation start site may play important roles in the activity of the cotton \textit{FT} promoter.

**Figure 4.** Repeat sequences in the 1.8-kb cotton \textit{FT} promoter in \textit{G. hirsutum}, \textit{G. arboretum}, and \textit{G. raimondii}. Harr-plot was used and the black arrow points downstream of the promoter.

### 2.5. Histochemical Activity of the 1.8-kb Sequence Upstream of the FT Promoter in Cotton

We hypothesized that the proximal cotton \textit{FT} promoter (−1 to −1000 bp) may play important roles in \textit{FT} regulation. To validate this hypothesis, two deletion constructs were generated (Figure 5A). The 1.0- and 1.8-kb \textit{FT} promoters were independently fused to GUS to generate various \textit{pFT:GUS} constructs, and they were stably transformed into Col-0 using \textit{Agrobacterium}. GUS-staining assays in \textit{Arabidopsis} showed that the strong GUS signals were induced by both 1.0- and 1.8-kb sequences upstream of cotton \textit{FT}. Furthermore, the activity level of the GUS reporter induced by the 1.0-kb transgenic plants was stronger than in the 1.8-kb transgenic plants; however, there was no difference in the GUS signal induced by the A and D subgenomes’ 1.0-kb \textit{FT} promoter fragments (Figure 5B). A gene transcription analysis using quantitative real-time PCR (qRT-PCR) showed that the expression levels of \textit{GUS} induced by both the 1.0- and 1.8-kb \textit{FT} promoters were consistent with the observed GUS signaling levels (Figure 5C).
Figure 5. Histochemical activity analyses of the 1.8-kb sequence upstream of cotton FT promoters. (A) Truncation of the cotton FT promoters of G. hirsutum, G. arboretum, and G. raimondii. RS represents the domain of enriched repeat sequences. (B) The histochemical analysis of GUS activity in the leaves of Arabidopsis growing on flowering day; (C) The expression level of the GUS gene in the transgenic Arabidopsis and wild type plants (Col-0) by qRT-PCR. Total RNA was extracted from the true leave tissues of Arabidopsis on flowering day. Data represent the mean ± SE (n = 3) obtained from three biological replicates. Statistically significant differences are indicated by different lower-case letters (p < 0.05, Duncan’s multiple range tests).

2.6. Functional Analysis of the 1.8-kb Sequence Upstream of the FT Promoter in Cotton

To further determine whether regulatory elements exist in the 1.8-kb sequence upstream of the cotton FT translation start site, we next generated transgenic ft-10 mutant Arabidopsis harboring 1.0- or 1.8-kb promoter fragments fused to the cDNA of FT. Consistent with the reporter gene assays, complementation analyses revealed that these constructs could partially rescue the late-flowering phenotype of ft-10 plants grown under LD conditions, and the 1.0-kb FT promoter fragments were more
efficient in rescuing the late-flowering phenotype than the 1.8-kb promoter fragments (Figure 6A,B) in transgenic Arabidopsis. In addition, gene transcription analyses in Arabidopsis using qRT-PCR showed that the GhFT gene was overexpressed in all the transgenic ft-10 plants under LD conditions (Figure 6C). The data demonstrated that the 1.0-kb FT promoter fragment might play an important role in the activity levels of FT promoters in cotton, but the possibility of other regions having specific transcriptional regulatory functions was not excluded.

Figure 6. Functional analysis of the 1.8-kb sequence upstream of the FT promoter of G. hirsutum, G. arboretum, and G. raimondii using Arabidopsis as a model plant. (A) Appearance of 30 d Col-0, ft-10, FTpros:GhFT transgenic Arabidopsis lines grown under LD conditions. Scale bar, 2 cm. (B) Flowering time was measured as the rosette number of leaves per plant. (C) The expression level of GhFT was determined by qRT-PCR. Data represent the mean value obtained from three biological replicates. Statistically significant differences are indicated by different lower-case letters ($p < 0.05$, Duncan’s multiple range tests).

3. Discussion

Cotton is an important economic crop, and upland cotton is the most widely planted species in the world [35]. A whole genome-wide analysis showed that there was only one homoeologous FT gene pair in the cotton genome, and the FT-homologue from G. hirsutum promotes early flowering in A. thaliana and Nicotiana tabacum, providing a good foundation for studying the molecular mechanisms of flowering regulation in cotton [24,25,28,36–38]. In this study, we studied the promoters of GhFT
from the A and D subgenomes of *G. hirsutum*, *GaFT* from the A2 genome of *G. arboreum* and *GrFT* from the D5 genome of *G. raimondii*.

3.1. Cotton FT Promoter Activity Levels Vary with Length and Origin

The promoter functions of the FT gene have been studied in several important crops, such as soybean [21], rice [22], and wheat [23], and the results suggested that the promoter activity varies among the different species. In this study, we identified the 1.8-kb promoters of *GhFT-A*, *GhFT-D*, *GaFT*, and *GrFT*, and a phylogenetic analysis classified the four promoters into two types, which corresponded to their genomic origins (Figure 3). A multiple sequence alignment revealed five distinct deletion fragments in the 5.9-kb cotton promoter (Figure 1). A large number of putative plant cis-elements were located in the proximal cotton end, such as the TATA-box and CAAT-box, for transcription initiation. In addition, some elements were found to be associated with hormones, light, stress tolerance, tissue expression, and circadian rhythm regulation (Tables S1 and S2).

The binding site CCAAT box of CO and NF-Y transcription factor are located approximately 5.3-kb upstream of FT in *Arabidopsis*, whereas the CORE1 and CORE2 sites are located between −220- and −161-bp upstream of the start codon [12]. Functional analyses of the *Arabidopsis* FT promoter indicated that the distal CCAAT element and the proximal CORE sites can regulate FT gene expression to control flowering transition. In this study, we analyzed the CORE elements and CCAAT domain of the cotton 1.8-kb *GhFT* promoter and found that they are all located 1.0-kb upstream of the promoter (Figure S1A,B), indicating that the 1.0-kb cotton FT promoter may contain the region responsible for activity, but the roles of other regions were not excluded. The CORE elements and CCAAT domain play key roles in the activation of the FT promoter in *Arabidopsis*, and the promoter has no activity if any of these motifs are deficient [12,16,18]. In addition, a number of repetitive fragments were found between the 1.1 and 1.4 kb in the *GhFT* promoters (Figure 4), which is an area that contained a large number of cis-regulatory elements. We hypothesized that this region may be a binding region of some unknown transcription factors and may also have an effect on the activity of the FT promoter. Therefore, we truncated the 1.8-kb FT promoter to 1.0 kb (Figure 5A).

GUS histochemical staining assays showed that the GUS activities induced by the 1.0-kb promoters of *GhFT-A*, *GhFT-D*, *GaFT*, and *GrFT* were stronger than their respective 1.8-kb promoters (Figure 5B,C), which indicated that the TGTG(N2-N3)ATG, and CCAAT domains might also determine the activity of the cotton FT promoter. Our study also demonstrated that there was no significant difference in promoter activities between the 1.0- or 1.8-kb promoter fragments of the A and D subgenomes using deletion constructs experiments. The promoter activity in the 1.8-kb fragment was weaker than that in the 1.0-kb fragment, implying that some unknown silencers may exist in the proximal cotton FT promoter region (−1.0 to −1.8 kb). A large number of repetitive sequences between 1.1- and 1.4-kb upstream of the FT translation start site were found (Figure 4), and we speculated that some unknown silencers are probably located in the repetitive enrichment region, which warrants further investigation.

Genetic complement assays showed that the 1.0-kb promoters of FT were more efficient in rescuing the late-flowering phenotype of *ft-10* plants than were the 1.8-kb promoters (Figure 6A), with similar rosette leaf numbers being obtained (Figure 6B). Furthermore, the bioinformatics analyses showed that the CORE and CCAAT domains existed in the 1.0-kb promoter of *GhFT*. Based on these experimental results, we speculate that the specific regulation of flowering transition, which involves CO and NF-Y, may exist in this region, but the influences of other regions were not excluded.

3.2. Photoperiod Sensitivities of the Promoter Regions from FT Homologues in Arabdiopsis and Cottons Are Different

In *Arabidopsis*, under LD inductive conditions, transcriptional activation of FT is directly mediated by CO, which has two responsive elements, CORE1 and CORE2, in the FT promoter that share the consensus sequence TGTG(N2-3)ATG [1,9]. The CCAAT-box binding site of CO and NF-Y transcription factors exist approximately 5.3-kb upstream of FT in *Arabidopsis*. Rice is a short-day (SD) plant, and
the CCAAT box of the potential cis-element in the Hd3a promoter did not contain any nucleotide changes compared with in Arabidopsis [11,22]. The rice Hd1 gene, which is an orthologue of the Arabidopsis CO gene, suppresses the transcriptional activation of Hd3a and inhibits flowering under LD conditions. In contrast, Hd1 gene activates Hd3a expression, causing the promotion of flowering under SD conditions [15,39]. Wild cotton is a perennial plant, and most species respond to short day-photoperiods, resulting in a variety of plant types and different flowering time. However, domesticated cotton varieties have undergone extensive artificial selection, resulting in their gradual loss in photoperiodism [27]. Our research revealed that in upland cotton, CORE and CCAAT domains were concentrated in the 1.0-kb region upstream of the translation start site (Figure S1A,B), which was different from in Arabidopsis [12]. Compared with the consensus sequence TGTG(N2-3)ATG, the CORE in the cotton FT promoter is atypical TGTG(N2-3)ATG. We speculate that CO homolog may bind to the atypical TGTG(N2-3)ATG in upland cotton, but it remains to be confirmed in future. However, whether the FT/CO regulatory model in upland cotton is conserved, as in Arabidopsis, remains to be further studied.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

G. hirsutum cv. Xinlucao 42, G. raimondii, and G. arboreum were field-grown during the summer of 2016 in Shihezi (Xinjiang, China) under natural conditions. The seeds of the mutant ft-10 (in the Col-0 background) were ordered from the Arabidopsis Biology Resources Center (ABRC, Columbus, OH, USA). The methods of seed sterilization and cultivation for ft-10 and A. thaliana Col-0, as well as the seedling growth conditions, used in this study were published previously [23,24]. For gene expression analyses, the fresh leaves of Col-0, ft-10, and the transgenic lines growing for 20 d were harvested under LD conditions. All the samples were frozen immediately in liquid nitrogen and stored at −80 °C until used.

4.2. Phylogenetic Analysis

The genomic sequences of FTs from G. hirsutum, G. arboretum, and G. raimondii were assembled using a batch Basic Local Alignment Search Tool (BLAST) search of shotgun sequences obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). mVISTA was used to perform pairwise alignments (http://genome.lbl.gov/vista) [40,41]. The alignment of the nucleotide sequences and the phylogeny reconstruction analysis of the FT promoter in cotton were performed as previously described [42–44].

4.3. Cloning of the FT Promoter, Vector Construction, and Transformation of Arabidopsis

The cotton FT promoter, extending approximately 1.0- and 1.8-kb upstream of the ATG, was cloned by PCR amplification using gene-specific primers designed based on the putative sequences in the G. hirsutum, G. arboreum, and G. raimondii genome database (Table S3) and then ligated to the pJET 1.2 blunt vector following the manufacturer’s instructions. The PlantCare database (http://bioinformatics.psb.ugent.be/webtools/plant-care/html/) was used to identify potential cis-regulatory elements. To analyze the RSs of the FT promoters, we used the Nucleic Acid Dot Plots website (https://en.vectorbuilder.com/tool/nucleic-acid-dot-plot.html) and entered the promoters as required. In the resulting figure, repetitive sequences were represented by point-intensive areas and were calculated according to the values on the coordinate axis. Then, the corresponding FT promoters, which were corrected by sequencing, and the GhFT gene of 35S:FT [24] were digested by restriction enzymes to independently replace the CaMV 35S promoter and GUS gene of pCAMBIA 1301 to generate pGhFT:FT constructs. The resulting constructs were transformed into Agrobacterium tumefaciens GV3101, which was used to transform Col-0 and ft-10, as previously described [45]. Kanamycin-resistant transformants were selected, and homozygotes were replanted and subsequently monitored for flowering using
non-transgenic \(\beta\)-10 seedlings as controls. Flowering time was assessed using the number of rosette leaves per plant at the time of the first flower bloomed.

To evaluate the activity of the \(\text{GhFT}\) promoter, \(\text{pGhFT}:\text{GUS}\) was constructed and transformed into \(A.\ thaliana\) (Col-0) to generate independent transgenic lines. The activities of \(\text{GhFT}\) promoters were detected by histochemical GUS staining. Fresh plant tissues were harvested and immersed in GUS staining solution [0.1 M \(\text{Na}_2\text{HPO}_4\cdot12\text{H}_2\text{O}\), 0.1 M \(\text{NaH}_2\text{PO}_4\cdot2\text{H}_2\text{O}\), pH 7.0, 0.1% Triton X-100, 5 mM \(\text{K}_3[\text{Fe(CN)}_6]\), 5 mM \(\text{K}_4[\text{Fe(CN)}_6]\cdot3\text{H}_2\text{O}\), 10 mM EDTA-\(\text{Na}_2\), pH 7.0 and 1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid]. After incubation at 37 \(^\circ\)C overnight, the plant materials were rinsed with 70% ethanol. The resulting stained tissues were observed under a microscope.

4.4. Gene Expression Analysis

Total RNA for each sample was isolated using the RNAprep pure Plant Kit (Tiangen, Beijing, China) and treated with RNase-free DNase according to the manufacturer’s protocol. The quality, quantity, and integrity of the total RNA extracted were assessed as previously described [24]. cDNA was synthesized from 200 ng RNA using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. qRT-PCR was carried out using the SYBR Green Master Mixture (CWBO, Beijing, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Foster City, CA, USA). The PCR amplification system and program were as described previously [14]. Three biological replications were performed with independently isolated RNA in all the qRT-PCR assays. Relative gene expression levels were calculated using the \(2^{-\Delta\Delta\text{Ct}}\) method [46].

4.5. Statistical Analysis

The results were based on three independent experiments with at least three replicates. The SPSS software package (ver.17.0; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis, as previously described [47].

5. Conclusions

In this study, we identified a 5.9-kb promoter of \(\text{FT}\) from \(G.\ hirsutum\) and revealed that the activity level of the truncated \(\text{GhFT}\) promoter varied in different tissues. Further studies on the 1.0- and 1.8-kb fragments of the \(\text{FT}\) promoters in \(G.\ hirsutum\), \(G.\ arboreum\), and \(G.\ raimondii\) showed that the activity of the 1.0-kb \(\text{FT}\) promoter was higher than that of the 1.8-kb promoter. These results illustrate that the proximal \(\text{FT}\) promoter fragment might play an important role affecting the activity levels of \(\text{FT}\) promoters in cotton flowering.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/19/4769/s1.

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