

# Molecular cloning and functional analysis of the FLOWERING LOCUS T (FT) homolog GhFT1 from *Gossypium hirsutum*

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**Abstract** FLOWERING LOCUS T (FT) encodes a member of the phosphatidylethanolamine-binding protein (PEBP) family that functions as the mobile floral signal, playing an important role in regulating the floral transition in angiosperms. We isolated an FT-homolog (GhFT1) from *Gossypium hirsutum* L. cultivar, Xinluzao 33. GhFT1 was predominantly expressed in stamens and sepals, and had a relatively higher expression level during the initiation stage of fiber development. GhFT1 mRNA displayed diurnal oscillations in both long-day and short-day condition, suggesting that the expression of this gene may be under the control of the circadian clock. Subcellular analysis revealed that GhFT1 protein located in the cytoplasm and nucleus. Ectopic expression of GhFT1 in transgenic arabidopsis plants resulted in early flowering compared with wild-type plants. In addition, ectopic expression of GhFT1 in arabidopsis ft-10 mutants partially rescued the extremely late flowering phenotype. Finally, several

flowering related genes functioning downstream of AtFT were highly upregulated in the 35S::GhFT1 transgenic arabidopsis plants. In summary, GhFT1 is an FT-homologous gene in cotton that regulates flower transition similar to its orthologs in other plant species and thus it may be a candidate target for promoting early maturation in cotton breeding.

**Keywords:** Cotton; floral transition; florigen; flowering time; FLOWERING LOCUS T

**Citation:** Guo D, Li C, Dong R, Li X, Xiao X, Huang X (2015) Molecular cloning and functional analysis of the FLOWERING LOCUS T (FT) homolog GhFT1 from *Gossypium hirsutum*. *J Integr Plant Biol* 57:522–533 doi: 10.1111/jipb.12316

**Edited by:** Stephan Wenkel, University of Tübingen, Germany

**Received** Oct. 22, 2014; **Accepted** Nov. 24, 2014

Available online on Nov. 27, 2014 at [www.wileyonlinelibrary.com/journal/jipb](http://www.wileyonlinelibrary.com/journal/jipb)

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## INTRODUCTION

In angiosperms, the timing of the transition from vegetative to reproductive growth stage is a fine-tuned process that is coordinately regulated through the integration of environmental cues and developmental signals. Among different indicators such as temperature and light, the plant's response to photoperiod is the most important factors for determining the right time to flower (Imazumi and Kay 2006). Early physiological experiments indicated that exposing a single leaf to inductive photoperiod was sufficient to induce flowering in plants (Naylor 1941) and provided a powerful support of the florigen hypothesis (Chailakhyan 1936).

The initiation of flowering in *Arabidopsis thaliana* is mainly controlled by five major pathways, including photoperiodic, vernalization, autonomous, gibberellin, and age pathways, which all converge on the integrator genes FLOWERING LOCUS T (FT) (Corbesier and Coupland 2006; Fornara et al. 2010). FT plays an important role in the control of flowering in *Arabidopsis*, which was identified as a causative gene for a typical late-flowering mutant ft (Kardailsky et al. 1999; Kobayashi et al. 1999). Recent advances in molecular biology have revealed that the gene products of FT in *Arabidopsis*, a long-day (LD) plant, and that of Heading date 3a (Hd3a) in rice, a short-day (SD) plant, and that of SINGLE FLOWER TRUSS (SFT) in tomato were elusive florigens (Lifschitz et al. 2006; Corbesier et al. 2007; Mathieu et al. 2007; Tamaki et al.

2007; Notaguchi et al. 2008). In *Arabidopsis*, the B-box zinc finger transcription factor CONSTANS (CO) induces the expression of FT and TWIN SISTER OF FT (TSF) in the vascular tissues under LD inductive conditions (Kardailsky et al. 1999; Kobayashi et al. 1999; Samach et al. 2000). FT and TSF proteins, produced in the phloem companion cells, are transported to the apex, where they are able to interact with FLOWERING LOCUS D (FD), a bZIP protein, to form one part of a complex, and then activate the expression of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) and the floral meristem identity genes APETALA1 (AP1) and LEAFY (Abe et al. 2005; Wigge et al. 2005; Yoo et al. 2005; Ho and Weigel 2014). Thus, FT/TSF constitutes the long-sought mobile floral signal molecule, florigen in *Arabidopsis* (Corbesier et al. 2007; Mathieu et al. 2007; Notaguchi et al. 2008). In rice, Hd3a interacts with its intracellular receptor 14-3-3 protein in shoot apical cells forming a complex; then translocates to the nucleus and binds to the OsFD1 to form a ternary “florigen activation complex” (FAC). The FAC induces transcription of OsMADS15, which leads to flowering (Taoka et al. 2011).

Because of duplication and divergence of ancestral genes, angiosperm genes containing a conserved phosphatidylethanolamine-binding (PEBP) domain have been shown to have the function diversification that fall into three clades (Chardon and Damerval 2005; Hedman et al. 2009; Karlgren et al. 2011). For example, there are six PEBP family proteins in *Arabidopsis* including FT and TSF, which define the FT-like clade,

TERMINAL FLOWER 1 (TFL1), BROTHER OF FT AND TFL1 (BFT), and ARABIDOPSIS THALIANA CENTRORADIALIS (ATC), which define the TFL1-like clade, and MOTHER OF FT AND TFL1 (MFT), which defines the MFT-like clade (Karlsgren et al. 2011). FT and TFL1 have antagonistic effects on flowering time, and their functions have been related to the presence of critical amino acid (AA) residues Tyr85/Gln140 in FT and His88/Asp144 in TFL1 (Hanzawa et al. 2005; Ahn et al. 2006). The substitution of specific AA can convert TFL1 into a floral inducer and FT into a floral repressor (Hanzawa et al. 2005; Ahn et al. 2006).

To date, FT-like genes that promote flowering have been identified in dicotyledonous plants such as tomato (Lifschitz et al. 2006), poplar (Böhnenius et al. 2006; Hsu et al. 2006), apple (Kotoda et al. 2010; Tränkner et al. 2010), sugar beet (Pin et al. 2010), cucurbits (Lin et al. 2007), sunflower (Blackman et al. 2010), pea (Hecht et al. 2011), soybean (Kong et al. 2010), potato (Navarro et al. 2011), tobacco (Harig et al. 2012), and fig (Ikegami et al. 2013), as well as in monocots such as rice (Kojima et al. 2002), barley (Faure et al. 2007), and maize (Danilevskaya et al. 2008). Therefore, the role of FT-like proteins in transmitting inductive signals in diverse plants suggests that it has a conserved ancestral function.

Flowering is not only a physiological phenomenon for plants but also an important trait for crops. The correct timing of flowering has adaptive value, and early flowering can shorten the life cycle of plants. Research on the molecular mechanism of flowering time has long been the focus from scientists to crops breeders. Cotton is not only one of the most valuable cash crop plants worldwide, but also an excellent model system for studying polyploidization, cell elongation, and cell wall biosynthesis (Wang et al. 2012). Cotton originated from a tropical region, and its growth is very sensitive to low temperature and soil conditions in temperate cultivation regions. Flowering earliness is an important objective in most cotton breeding programs (Godoy and Palomo 1999; Argiriou et al. 2008). The cotton genus (*Gossypium*) contains 50 species and five allopolyploid species (Wendel and Albert 1992), and different *Gossypium* genus show diverse flowering time and temperature-sensitive response. It is therefore necessary to understand how flowering is regulated in such an important textile fiber crop, where the genetic basis of flowering has received little attention. Isolation of genes that regulate the flowering switch in cotton will facilitate in understanding the mechanisms of aging, flowering, and eventually the mechanisms to increase the fiber yield of cotton.

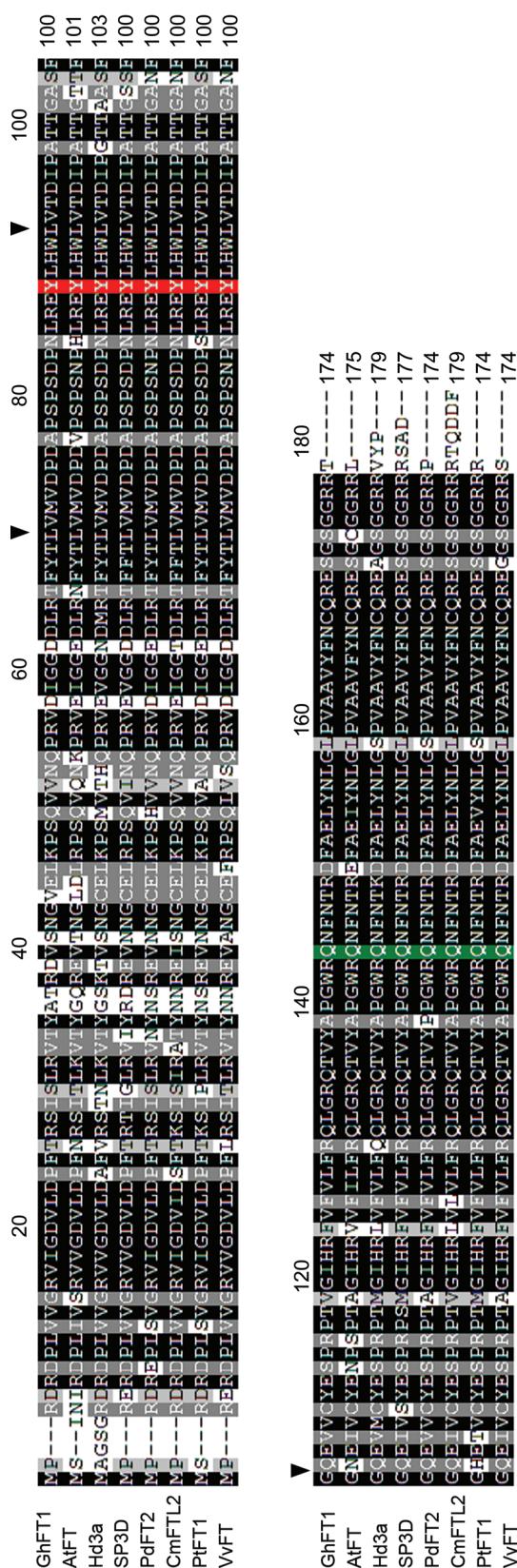
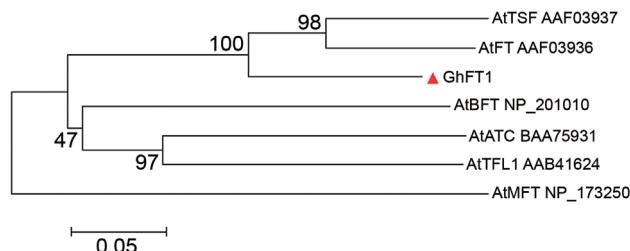
Argiriou et al (2008) isolated two TFL1 homologs (*GhTFL1a* and *GhTFL1b*) from *Gossypium hirsutum* L. Expression analysis revealed that the genes of *GhTFL1a* and *GhTFL1b* have slightly different expression patterns. However, the exact biological functions of *GhTFL1a* and *GhTFL1b* in the determination of meristem fate remain unclear. In this study, we cloned the first known FT-like gene from cotton, *GhFT1*. We studied its expression profiles in tissue specificity, and diurnal rhythmicity. We characterized its roles by ectopic expression of *GhFT1* in wild-type (WT) *Arabidopsis*, and loss-of-function mutation in *FT* plants. We found that *GhFT1* plays an important role in regulating flowering time, and may have potential in the regulation of fiber development. This work provides a foundation for exploring the roles of the *FT* family members in cotton developmental regulation.

## RESULTS

### Isolation and characterization of an *FT*-like gene from *G. hirsutum*

To isolate genes encoding *FT*-like family proteins from cotton, a cloning strategy combining bioinformatic analysis and rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) was used. One *FT* homolog designated *GhFT1* (GenBank accession no. HM631972) was isolated from *G. hirsutum*. Comparison of the deduced AA sequences deposited in GenBank indicated that *GhFT1* shared a high identity with *FT* of other plants, such as 77%, 82.8%, 87.9%, 90.8%, 89.7%, 89.7%, and 89.1% identity with *Arabidopsis FT*, rice *Hd3a*, tomato (*Solanum lycopersicum*) *SP3D*, eastern cottonwood poplar (*Poplar deltoides*) *PdFT2*, Cucurbits (*Cucurbita maxima*) *CmFTL2*, black cottonwood (*Poplar tremula*) *PtFT1*, and grapevine (*Vitis vinifera*) *VvFT*, respectively (Figure 1). Phylogenetic tree analysis between *GhFT1* and six *Arabidopsis PEBP* family members indicated that *GhFT1* has the closest kinship relationship with *AtFT*-like (Figure 2). Genomic structural analysis revealed that the *GhFT1* gene was 3,151 bp long and consisted of four exons with three introns at conserved positions identical to those in *FT* genes from other species (Figure S1A). The open reading frame (ORF) of *GhFT1* is 525 bp encoding a protein of 174 AA. Two whole genome shotgun sequences were acquired using *GhFT1* cDNA as the query against the *Gossypium raimondii* (D5) and *Gossypium arboreum* (A2) genome sequence database released recently (Paterson et al. 2012; Wang et al. 2012; Li et al. 2014), respectively. Comparison of cDNA and genome sequences confirmed that the *GaFT1* gene has 3,131 bp containing four exons and three introns, and the coding region of which contains a 525 bp ORF encoding 174 AA. Accordingly, the *GrFT1* gene has 3,145 bp containing four exons and three introns, and the coding region of which contains a 525 bp ORF encoding 174 AA. Amino acid alignment among *GhFT1*, *GaFT1*, and *GrFT1* indicated no differences at the protein level, and the main differences at the nucleotide level lie in the part of introns (Figure S1A). An extended version of multiple AA alignment including *GhFT1* and other characterized *FT/TFL1* homologs is shown in Figure S1. The conserved key AA residues Tyr88 (Y) and Gln144 (Q) in *FT*-like proteins were identified in the *GhFT1* protein (Figures 1, S1B), which are critical for *FT* activity, and these residues are diagnostic AA that distinguish *FT*-like floral promoters from *TFL1*-like floral repressors (Hanzawa et al. 2005; Ahn et al. 2006). The conserved domains LGRQTVYAPGWRQN (14 AA) known as “segment B” and LYN/IYN in “segment C” in *GhFT1* were similar to the other *FT* proteins (Figures 1, S1B).

Forty-nine AA sequences belonging to the *PEBP* family (MFT-like, FT-like, and TFL1-like) representing monocots and dicots were selected to construct an expanded phylogenetic tree (Figure S2). The multiple AA alignments showed that these 49 *PEBP* homologs shared high similarity to each other, ranging 75%–92.5%. Among them, the highest identity was found to have 92.5% similarity with *CpFT* (*Carica papaya*) and *FcFT* (*Ficus carica*). Phylogenetic analysis showed that *GhFT1* formed a clade with the most closely related plant *FT*-like homologs, and *GhFT1* was mainly grouped with the dicotyledonous Rosales *FT* subgroup (Figure S2). This grouping suggests *GhFT1* may promote flowering.

**Figure 1. Continued.****Figure 2. Phylogenetic analysis of cotton GhFT1 and PEBP family in *Arabidopsis***

Amino acid sequences of the GhFT1 and six PEBP proteins from *Arabidopsis* were aligned using Clustal W. The tree was constructed by the neighbor-joining (N-J) method. Bootstrap values for 1,000 resamplings are shown on each branch. The unit for the scale bar displays branch lengths (0.05 substitutions/site). Accession numbers are indicated at the right of the protein name. Red triangle represents the GhFT1.

#### Analysis of GhFT1 expression in cotton

The transition from vegetative to reproductive growth is mainly controlled by day length in many plant species. Day length is perceived by leaf and induced a systemic signal, called florigen, which moves through the phloem to the shoot apex (Corbesier et al. 2007; Lin et al. 2007; Mathieu et al. 2007). Many FT-like homologs are expressed exclusively in leaf, such as BvFT1 and BvFT2 in sugar beet (*Beta vulgaris*) (Pin et al. 2010), and NtFT1-NtFT4 in tobacco (*Nicotiana tabacum*) (Harig et al. 2012). We first analyzed their expression patterns in young leaves as well as other different tissues of cotton by quantitative real-time reverse transcription (qRT)-PCR. GhFT1 mRNA was detected in all the investigated samples with big differences in their expression levels (Figure 3A). GhFT1 were more highly expressed in stamens, sepals, petal, carpel, and fiber than in root, stem, leaf, and ovule. Our results showed that the expression pattern of GhFT1 was different from that of GhTFL1a and GhTFL1b, which were present mainly in the root and secondary in leaf shoot apical meristem and flower (Argiriou et al. 2008). In fact, the expression pattern of GhFT1 was very similar to the expression pattern of FT in *Arabidopsis*.

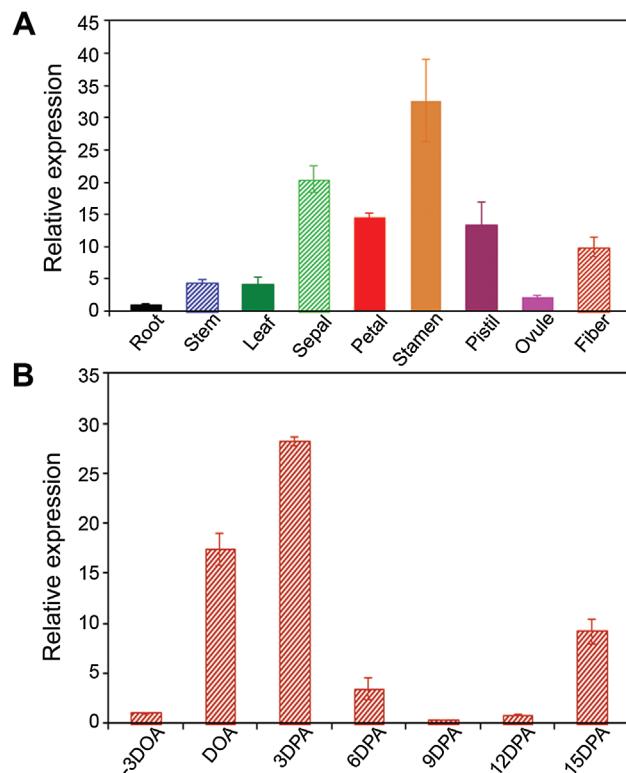
#### Figure 1. Sequence comparison of GhFT1 and related FT-like proteins

Alignment of amino acid sequences of GhFT1 and FT-like proteins from other plant species, including *Arabidopsis* (*Arabidopsis thaliana*: FT, AAF03936), rice (*Oryza sativa*: Hd3a, BAB61028), tomato (*Solanum lycopersicum*: SP3D, AAO31792), poplar (*Populus deltoides*: PdFT2, AAS00056; Poplar tremula: PtFT1, ABD52003), Cucurbita (*Cucurbita maxima*: CmTFL2, ABI94606), and grapevine (*Vitis vinifera*: VvFT, ABF26526). Amino acids in black and gray are identical and similar, respectively. The gaps indicated by dashes are attributed to the lack of amino acids. Amino acids shown in pink and green indicate the two highly conserved amino acid Tyr (Y) and Gln (Q) for FT orthologs, respectively. The black inverted triangle indicates the position of the intron.

AtFT was expressed in all the tissues in seedlings and mature plants. In mature plants, the AtFT mRNA was present in the whole seedling, shoot apex, floral buds, flowers, siliques, stems, rosette leaves, bract, and root. However, AtFT showed a higher expression level in flowers, and immature siliques (Kobayashi et al. 1999).

To detect mRNA level of GhFT1 gene during the developmental stages of cotton fiber, the samples were collected from -3 d of anthesis (DOA) to 20 d post-anthesis (DPA) every 3 d. The results of qRT-PCR revealed that the expression levels of GhFT1 were highest during the fiber initiation stage from -3 DOA to 3 DPA and peaked at 3 DPA. GhFT1 transcript levels dropped during the fiber elongation stag from 6 to 12 DPA, and then gradually increased again from the secondary cell wall deposition stage in 15 DPA (Figure 3B).

To explore whether the expression of GhFT1 was influenced by daily oscillations in photoperiod, the level of GhFT1 transcription was analyzed every 4 h over a 48 h period in 20 d old plants growing in LD and SD conditions using qRT-PCR,



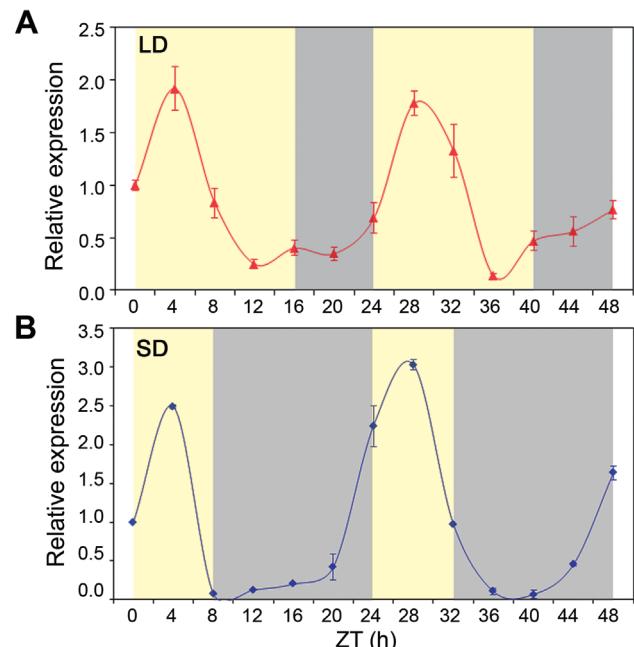
**Figure 3. Expression pattern of GhFT1 in cotton different tissues**

(A) Expression patterns of GhFT1 in various tissues, including root, stem, leaf, sepal, petal, stamen, pistil, ovule, and fibers. Roots, stem, and leaf were sampled at the third true leaf stage, and other tissues samples were collected on the same day during the flowering stage. Ovules and fibers were sampled 15 d post-anthesis (DPA), respectively. Data represent the mean  $\pm$  SE ( $n=3$ ). (B) GhFT1 expression pattern in various fiber development periods of cotton. Data represent the mean  $\pm$  SE ( $n=3$ ). DOA, d of anthesis.

respectively. GhFT1 expression was similar under LD and SD with a rhythmic cycle. The expression of GhFT1 in *G. hirsutum* plants showed diurnal oscillations (Figure 4). These results indicated that the mRNA level of GhFT1 obviously increased during 4 h of the light period with peak at zeitgeber time (ZT) 4, and quickly decreased with its lowest expression at ZT 12, and then gradually rose during the night. The results indicated that a diurnal pattern for GhFT1 transcription does not follow the usual pattern. For example, in contrast to *Arabidopsis* AtFT mRNA with a peak at dusk (Yamaguchi et al. 2005; Corbesier et al. 2007), GhFT1 transcript peaked in the morning.

#### Cytoplasm and nucleus location of GhFT1 protein

To elucidate the cellular location of GhFT1, we fused green fluorescence protein (GFP) to the C-terminus of GhFT1 protein expressed under the 35 S promoter of the Cauliflower Mosaic Virus (CaMV), and the resulting construct of 35S::GhFT1-GFP was then transformed stably into *Arabidopsis* (Col-0). The subcellular location of the fusion protein was observed by confocal imaging of green fluorescence in the cells of transgenic seedlings. As shown in Figures 5 and S3, green fluorescence was found in both the cytoplasm and nucleus in



**Figure 4. Detection of diurnal expression of GhFT1 by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in long-day (LD) and short-day (SD) conditions**

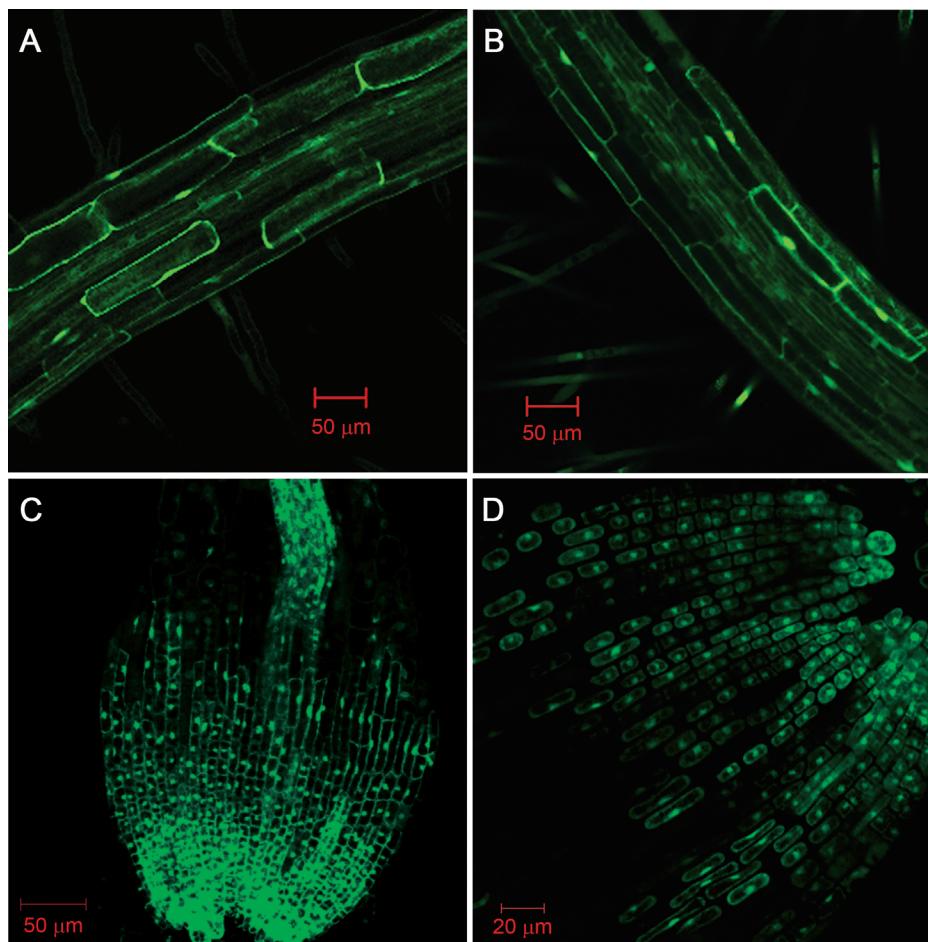
(A) Diurnal expression profile of GhFT1 from cotton under LD (16 h light/8 h dark) condition at zeitgeber time (ZT). (B) Diurnal expression profile of GhFT1 from cotton under SD condition (8 h light/16 h dark) at ZT. Total RNA was isolated and analyzed from the third true leaf every 4 h. Sample collection started at the beginning of the light period (time 0) and continued every 4 h for 48 h in LD and SD conditions, respectively. Each experiment was repeated three times with similar results. Light yellow boxes indicate day, and gray boxes, night. Data represent the mean  $\pm$  SE ( $n=3$ ).

root cells of transgenic *Arabidopsis* plants expressing GhFT1-GFP (Figure 5B, C), which was similar to that of transgenic seedling expressing GFP alone (Figure 5A). To exclude the possibility of cell wall association of GhFT1-GFP, the root cells were plasmolyzed by sucrose treatment. As shown in Figure 5D, the fluorescence in the plasmolysis root tip cells was detached from the cell wall, and internalized, indicating a cytoplasm rather than a cell wall localization of GhFT1-GFP fusion protein. This observation suggests that GhFT1 localized in both the cytoplasm and nucleus in plant cells, which was similar to the AtFT, Hd3a, and NtFT proteins (Taoka et al. 2011; Harig et al. 2012).

#### Ectopic expression of GhFT1 promotes flowering in *Arabidopsis*

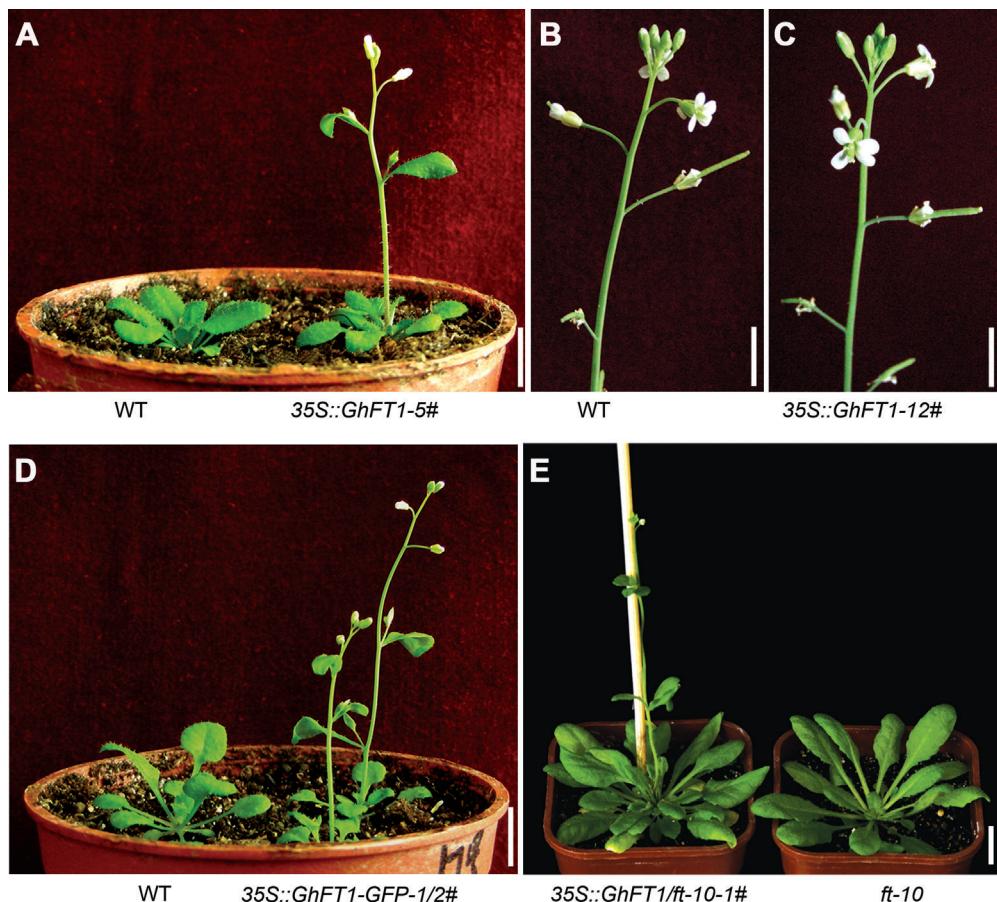
To explore the potential of GhFT1 in the regulation of flowering, this gene was overexpressed in Col-0 using the

CaMV 35 S promoter by transformation with 35S::GhFT1. As a result, 17 independent 35S::GhFT1 transgenic lines expressing GhFT1 cDNA were generated, and all the transgenic plants showed identical phenotypes by flowering earlier than WT plants. In the homozygous T<sub>3</sub> plants, 12 were significantly early flowering phenotypes under LD, and five also showed precocious flowering compared with WT plants under SD conditions, relative to WT (Figure 6). Under LD conditions, the 35S::GhFT1 transgenic Col-0 plants reached anthesis 6 d earlier, and with five fewer leaves, than the WT controls (Figure 6A; Table 1). Furthermore, all transgenic plants showed a normal growth habit, produced indeterminate inflorescences like WT plants, fertile flowers, normal siliques, and germinating seeds (Figure 6B, C). Under SD, these 35S::GhFT1 transgenic plants flowered approximately 24 d after sowing by producing approximately nine rosette leaves (Table 1; Figure S4). To investigate whether the early flowering phenotype correlated



**Figure 5. Nucleus and cytoplasm subcellular location of GhFT1-green fluorescent protein (GFP) in the root cells of transgenic *Arabidopsis* plant**

(A) Green fluorescent protein image of hypocotyl of 35S::GFP transgenic plants. (B) Green fluorescent protein image of hypocotyl of 35S::GhFT1-GFP transgenic plant. (C) Green fluorescent protein image of root tip of 35S::GhFT1-GFP transgenic plant. (D) Green fluorescent protein image of root tip of 35S::GhFT1-GFP transgenic plant after 20% (w/v) sucrose treatment for 10 min. *Arabidopsis* (Col-0) were transformed with 35S::GFP and 35S::GhFT1-GFP constructs, respectively. The T<sub>1</sub> transgenic plants expressing GFP and GhFT1-GFP were observed under a confocal microscope, respectively.



**Figure 6. Phenotype analysis of transgenic *Arabidopsis* lines that ectopically expressed *GhFT1***

(A) Appearance of 17 d wild-type *Arabidopsis* (Col-0, left) and transgenic *Arabidopsis* with 35S::*GhFT1* (right, transgenic line 5) grown in growth chamber under long-day (LD) conditions. (B) Appearance of flowers and siliques in Col-0. (C) Appearance of flowers and siliques in transgenic plants with 35S::*GhFT1*. Plant represents transgenic line 12. (D) Appearance of wild-type *Arabidopsis* (Col-0, left), transgenic *Arabidopsis* with 35S::*GhFT1*-GFP (right, transgenic line 1 and 2) 18 d after transfer to the growth chamber under LD condition. Scale bar, 1 cm. (E) Phenotype of 38 d *GhFT1* expression complements the late flowering phenotype of the *ft-10* mutant under long-day condition. Plant represents transgenic line 1.

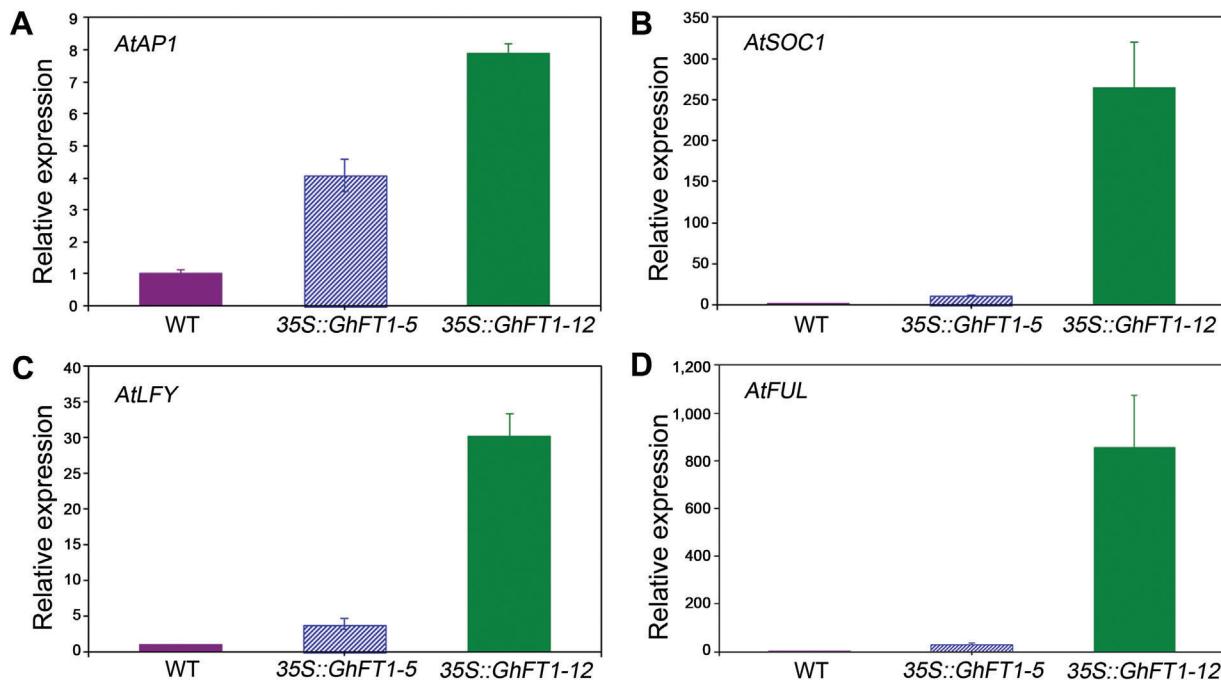
with *GhFT1* expression in 35S::*GhFT1* transgenic plants, qRT-PCR analysis was performed. As shown in Figure S5, higher *GhFT1* expression was observed in the extreme early flowering plants of 35S::*GhFT1* more than in the transgenic

plants with a less or WT phenotype. Multiple flower meristem identity genes, for example, *AtAP1*, *AtSOC1*, *AtLFY*, and *AtFUL*, and were also upregulated in the transgenic plants (Figure 7).

**Table 1. Overexpression of *GhFT1* in *Arabidopsis thaliana* Col-0 accession and mutant plants**

Genotype	Light conditions	No. of plants	Time of anthesis (d)	Rosette leaf number formed at anthesis
Col-0	LD	12	23.3 ± 0.9	12.4 ± 1.0
35S:: <i>GhFT1</i>	LD	12	17.1 ± 0.7**	7.3 ± 0.9**
<i>ft-10</i>	LD	6	63.0 ± 5.1	37.5 ± 3.4
35S:: <i>GhFT1</i> / <i>ft-10</i>	LD	12	39.7 ± 3.7**	25.2 ± 1.6**
35S:: <i>GhFT1</i> -GFP	LD	10	18.8 ± 0.8**	8.7 ± 0.6**
Col-0	SD	9	38.7 ± 0.7	13 ± 1.4
35S:: <i>GhFT1</i>	SD	5	24.6 ± 1.5**	8.5 ± 1.3**

\*\* and \* indicate significant differences at  $P < 0.01$  and  $P < 0.05$ , respectively, according to Student's t-test compared with wild type or mutant. LD, long-day (16 h light/8 h dark); SD, short-day (8 h light/16 h dark). Values are the mean ± SD from individual plants.



**Figure 7. *Arabidopsis* endogenous flowering-related gene expression patterns in wild-type (WT) and transgenic line 5 and 12 expressing *GhFT1***

Data represent the mean  $\pm$  SE ( $n = 3$ ). AtAP1, NM\_105581; AtSOC1, NM\_130128; AtLFY, NM\_125579; AtFUL, NM\_12584.

We also generated 10 independent 35S::GhFT1-GFP transgenic plants. Under LD, these transgenic plants flowered at 18.8 d after sowing by producing 8.7 rosette leaves (Figure 6D; Table 1). These results strongly indicated that the gene product of GhFT1 functions as a floral activator to regulate flowering.

#### Ectopic expression of *GhFT1* partially restored *ft-10* mutant phenotypes in *Arabidopsis*

*Arabidopsis thaliana* *ft-10* is a loss-of-function mutant of *FT* isolated from a T-DNA library, in which a T-DNA is inserted in the first intron of *FT* (Hanzawa et al. 2005), showing a strong late flowering phenotype under an LD condition (40.6 leaves vs 15.0 leaves for WT) (Yoo et al. 2005).

To further confirm whether GhFT1 could compensate for the *FT* function in *Arabidopsis*, the 35S::GhFT1 constructs were introduced into the late-flowering *ft-10* mutant. Five independent transgenic plants showed different degrees of flowering earlier than *ft-10* mutants under LD (Figure 6E). The average flowering time for these 35S::GhFT1/*ft-10* transgenic plants is approximately 40 d after sowing by producing 25 rosette leaves (Table 1). The flowering of *ft-10* mutants occurred at approximately 63 d and more than 37 rosette leaves were produced. However, the average flowering time for these 35S::GhFT1/*ft-10* transgenic plants was still approximately 16 d later than that of WT plants (Table 1). This result indicates that although the function of cotton GhFT1 is similar to that of *Arabidopsis FT*, it is still not able to completely complement *Arabidopsis FT* in flowering time regulation.

## DISCUSSION

#### Cloning and characterization of *GhFT1*

A number of studies indicated that *FT* homologs, in different plant species, have been shown to play a universal role in the regulation of flowering time. Conserved domain sequences within the *FT* proteins are required for the promotion of flowering. The Tyr85/His88 and Gln140/Asp144 residues, as well as sequences within the external loop of the PEBP proteins, known as segment B in exon 4, determine whether the *FT*-like and *TFL1*-like members promote or repress flowering (Hanzawa et al. 2005; Ahn et al. 2006).

To better understand the patterns and processes of homolog divergence in a young allopolyploid genome, we cloned an *FT*-like gene from cotton. Our results showed that the structure of *Gossypium* *FT* homologous gene contained three introns and four exons, which is similar to other species, such as *Arabidopsis*, rice, barley, grape, apple, poplar, maize, and sugar beet (Figure S1). The strong sequence identity to *FT*-homologous genes of other species and the presence of the *FT*-specific protein motifs revealed that the GhFT1 may be also involved in the control of flowering in cotton. Comparison of genome and AA sequences among GhFT1, GaFT1, and GrFT1 indicated that the locus was well conserved after tetraploidization (Figure S1).

#### Expression patterns of *GhFT1* in cotton

GhFT1 showed a broad expression pattern in different tissues: predominantly expressed in flower organs as well as in stems and leaves (Figure 3A), which is consistent with other *FT*

homologs but obviously different from *GhFTL1a* and *GhFTL1b* which mainly expressed in roots (Argiriou et al. 2008). FT-like homologs from different plant species are expressed exclusively in leaf tissues, but some also exhibit a broad expression pattern. For example, four tobacco FT-like genes *NtFT1-NtFT4* were expressed exclusively in leaf tissues under both LD and SD (Harig et al. 2012); and sugar beet contains two *FT* genes *BvFT1* and *BvFT2*, and expression of *BvFT1* and *BvFT2* was detected in various tissues, including seed, hypocotyl, cotyledon, and flower. However, *BvFT1* was mainly expressed in the leaf of juvenile stage, and *BvFT2* expressed mainly in the leaf of reproductive stage (Pin et al. 2010); *AtFT* expressed in all the tissues in seedling and mature plants, and showed higher expression level in flowers and immature siliques (Kobayashi et al. 1999). A broad expression pattern may suggest that *GhFT1* may play a diverse role in regulation of cotton development to environment cues. Nevertheless, the expression profile of *GhFT1* in leaf during different development stages will need to be further studied intensively.

Cotton seed fibers are highly elongated single-celled trichomes that differentiate from the outer epidermis of the ovule. Fiber development occurs in four distinct, but overlapping, stages: (i) initiation; (ii) elongation; (iii) secondary cell wall synthesis; and (iv) maturation (Basra and Malik 1984; Lee et al. 2007). The initiation stage starts from -3 d of anthesis (DOA) to 3 d post-anthesis (DPA), and is followed by the fiber elongation stage. In *G. hirsutum*, fibers develop prior to or on the day of anthesis (Lee et al. 2007). Floral transition and fiber initiation are two key events that eventually contribute to the production and quality of fiber. Our results showed that *GhFT1* transcript level was strongly upregulated in fiber cells during the fiber initiation and early elongation (-3 DOA and 6 DPA) (Figure 3B), but after that were gradually downregulated, which indicated that *GhFT1* may be involved in the process of fiber development, which will be further studied intensively. More research showed that *FT* orthologs from different plant species play diverse roles in the regulation of development. The expression profile indicated that *GhFT1* in leaves may confer flowering control, while in fibers contribute to fiber development, although much stronger evidence is needed.

The *GhFT1* transcript has a robust diurnal rhythm in both LD and SD conditions (Figure 4), suggesting that one mechanism may exist to mediate the light photoperiod induction of the *GhFT1*. Furthermore, similar to *FT* orthologs (Böhlenius et al. 2006; Hsu et al. 2006), *GhFT1* mRNA is also regulated by light with the level highest at the fourth to eighth hour of the light period and lowest at noon. This pattern, however, is different from *AtFT*, which has its level highest at the 16<sup>th</sup> hour of the light period and lowest at dawn under LD conditions (Yamaguchi et al. 2005; Corbesier et al. 2007). Cotton (*G. hirsutum*) is naturally a photoperiodic perennial that flowers under SD, but domesticated varieties that are managed as annual row-crops are day-neutral plants (McGarry and Ayre 2012; McGarry et al. 2013). The difference in the expression pattern for *FT* orthologs in response to light may be due to the fact that cotton (*G. hirsutum* var. Xinluzao 33) is a day-neutral plant which is a commercial upland cotton widely planted in Xinjiang, whereas *Arabidopsis* is a facultative LD plant.

### Subcellular location of *GhFT1-GFP*

Our study indicated that *GhFT1* is a cytoplasm and nucleus localization protein (Figures 5, S3) that is similar to *AtFT*, *Hd3a*, and *NtFTs-NtFT4* proteins, indicating that *GhFT1* may play a conserved role in the function of flowering regulation. In rice protoplasts, the fluorescence from *Hd3a* tagged with mCherry was also detected in both the cytoplasm and the nucleus. Crystallization as well as biochemical analysis *in vitro* indicated that 14-3-3 proteins act as intracellular receptor for *Hd3a* (florigen), which is transported from leaves to the shoot apex. Once *Hd3a* enters the cells in the shoot apex, it initially binds 14-3-3 proteins in the cytoplasm, and then the complex enters the nucleus to form a ternary complex with *OsFD* to activate transcription of *OsMADS15*, eventually leading to floral induction (Takao et al. 2011). Harig et al (2012) constructed the C-terminal fusion of *AtFT* and four tobacco *FT*-like proteins with the fluorescent reporter protein Venus under the control of the CaMV 35 S promoter. When constructs were expressed in *Nicotiana benthamiana*, the fluorescent signals were detected in the cytoplasm and nucleus of epidermal cells, respectively (Harig et al. 2012). Bimolecular fluorescence complementation (BiFC) showed that four tobacco *FT*-like proteins were able to interact with *Arabidopsis FD* exclusively in the nucleus in a similar manner to *Arabidopsis FT* (Wigge et al. 2005; Abe et al. 2005).

### Overexpression of *GhFT1* in the Columbia ecotype *Arabidopsis* and *ft-10* mutant

The transgenic plants carrying the 35S::*GhFT1* construct flowered earlier and had fewer rosette leaves at the time of flowering than the WT plant, indicating that its role in floral induction is conserved (Figure 6). Up to now, the roles of at least 23 *FT*-like genes from different plant species have been explored in heterologous and homologous plant systems (Table S2). The overexpression of *FT* homologous genes from different plants resulted in precocious flowering, assuming a conserved gene function in the regulation of flowering time. For example, transgenic *Arabidopsis* plants constitutively expressing *AtFT* or *AtTSF*, *GmFT2a* from soybean, and *PdFT2* from poplar flowered under LD and SD conditions after two or eight rosette leaves had been developed (Kardailsky et al. 1999; Kobayashi et al. 1999; Hsu et al. 2006; Sun et al. 2011). Amino acid sequence alignment between *GhFT1* and these 23 *FT* orthologs revealed that *GhFT1* has the smallest divergence with *MdFT2* (8.5; numbers indicate divergence times, that are calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign) and *PdFT2* (9.8), followed by *CmFTL2* (11.2) and *PtFT1* (11.2) (Table S3).

In addition, we observed that while 35S::*GhFT1/ft-10* transgenic plants flowered earlier than *ft-10* mutants, they still flowered later than WT plants (Figure 6E; Table 1). This indicates that the function of cotton ortholog *GhFT1* was not able to completely complement *Arabidopsis FT* in flowering time regulation, which resembles the function of the orchid ortholog, *OnFT* (Hou and Chang 2009). Florigen was produced in the leaf and transported to the shoot apex, where it forms a protein complex with bZIP transcription factors to regulate transcription of target genes, and then induces flowering. In rice shoot apical cells, the interaction between *OsFD* and *Hd3a* is not direct, but mediated by a 14-3-3 protein (Takao et al. 2011). Our results may be able to explain some of this

difference because FT function depends on the association with other factors. Further study will identify FD homologs in cotton to analyze whether GhFT1 interact with them in a similar manner with *Arabidopsis*, rice, and tobacco FT.

In *Arabidopsis*, rice, and tomato, it was shown that FT proteins move through the phloem from the leaves as the place of light perception to the shoot apex as the place of flower formation by detection of FT-GFP fusion protein in transgenic plants (Corbesier et al. 2007; Lifschitz et al. 2006; Tamaki et al. 2007). Introduction of 35S::GhFT1-GFP into WT plants caused these plants to flower much earlier than WT (Figure 6D). This result indicated that GhFT1-GFP promotes flowering, and it has similar activities with the WT FT protein.

More FT-like proteins have been identified as major regulators in a wide range of developmental process, such as fruit yield (Krieger et al. 2010), vegetative growth (Hsu et al. 2011), termination of sympodial meristems and leaf architecture (Shalit et al. 2009), tuberization (Navarro et al. 2011), the time of bud-set and bud-burst (Bohlenius et al. 2006; Gyllenstrand et al. 2007), and stomata guard cells opening (Kinoshita et al. 2011), which indicate that FT-like proteins should be recognized as mobile signals that can serve a pleiotropic function. When AtFT is ectopically overexpressed in cotton through virus-induced flowering (VIF), it uncouples flowering from photoperiodic regulation and promotes determinate growth in aerial organs (McGarry and Ayre 2012; McGarry et al. 2013). These findings further provide evidence that besides flowering hormone, florigen could also promote a more determinate habit in all aerial organs, supporting the idea of balance model, which argues that the balance of indeterminate and determinate growth is influenced by the relative ratio of indeterminate and determinate factors in the growing apices (Shalit et al. 2009; Krieger et al. 2010; McGarry and Ayre 2012; McGarry et al. 2013). Judicious manipulation of FT and related genes holds promise for enhanced cotton production (McGarry et al. 2013).

Although cotton is an extensively studied crop due to its economic and academic value, GhFT1 is the first FT-like gene isolated and characterized to date. In conclusion, this work provides a detailed expression and molecular characterization of the *Gossypium* FT gene and demonstrates that GhFT1 plays a conserved role in the regulation of flowering in cotton. Further research is needed to elucidate the biological function in cotton and to clarify its roles in the control of flowering and fiber development in this economically important crop plant for the textile industry.

## MATERIALS AND METHODS

### Plant materials and growth conditions

*Gossypium hirsutum* L. cultivar, Xinluzao 33, was kindly supplied by Professor Xueyuan Li from the Research Institute of Industrial Crops, Xinjiang Academy of Agricultural Sciences (Xinjiang, Urumqi, China). *Gossypium raimondii* (D5 genome) and *G. arboreum* (A2 genome) were kindly supplied by Professor Fang Liu from the National Wild Cotton Nursery (Hainan, Sanya, China). Cotton seeds were field-grown under natural conditions during the summer of 2013 in Shihezi (Xinjiang, China).

The seeds of *Arabidopsis thaliana* ecotype Col-0 and the mutant ft-10 (in the Col-0 background) obtained from the Arabidopsis Biology Resources Center (ABRC, Columbus, OH, USA) were surface sterilized for 20 min with 2.8% sodium hypochlorite solution containing 0.1% surfactant (Triton X-100; Sigma-Aldrich, Munich, Germany), rinsed several times with sterile water. Then, seeds were stratified for 3 d at 4 °C in darkness and then plated on the Petri dishes with half-strength Murashige-Skoog (MS) salt (pH 5.7; Duchefa, Haarlem, the Netherlands) mixture, 1% (w/v) sucrose, and 0.8% (w/v) agar. Petri dishes were then placed in the phytotron at 22 °C for 10 d, then the seedlings were transplanted to soil in a growth chamber under LD conditions (16 h light/8 h dark) or SD conditions (8 h light/16 h dark), and the light intensity for *Arabidopsis* growth is 200 μmol/m<sup>2</sup> per s.

For tissue expression analysis, roots, stems, and leaves were collected at the third true leaf expanding stage (20 d after planting). Bracts, sepals, petals, stamens, carpels, 15 DPA ovules, and 15 DPA fibers were collected during the cotton flowering period. Cotton bolls were harvested for gene expression analysis by qRT-PCR at the following time-points during development: -3 to 0 DPA ovules, 3–6 DPA ovules which contain initiating fiber cell, and 9–15 DPA fibers. Harvested bolls were placed on ice and transported to the laboratory and dissected. For diurnal rhythmic expression analysis, the plants were grown in a 25 °C chamber in LD (16 h light/8 h dark photoperiod) and SD conditions (8 h light/12 h dark photoperiod) with 150 μmol/m<sup>2</sup> per s of light intensity, respectively. The third true leaf (20 d after planting) was sampled every 4 h at 12 different time points from 06.30 hours for 2 d. All samples were frozen in liquid nitrogen and stored at -80 °C.

### Cloning GhFT1 from *G. hirsutum*

The AA sequence of AtFT (AAF03936) (Kardailsky et al. 1999) was used as a query sequence for tBLASTN (<http://www.ncbi.nlm.nih.gov/>) against cotton expressed sequence tag (EST) databases deposited in the National Center for Biotechnology Information. The EST (GenBank accession no. ES826802.1) was found with a predicted protein sequence with high identity to that of AtFT. This EST was then used as a template to design gene-specific primers. The 5'- and 3'-RACE kits (Clontech, Palo Alto, CA, USA) were used to amplify full-length cDNA according the manufacturer's instructions. The GhFT1-specific primers GhFT1-F1 and GhFT1-R1 were used to amplify the sequence of GhFT1. For 3'-RACE, the oligonucleotides included in the 3'-RACE kit were used as antisense primers, and gene-specific primers GhFT-3-F and GhFT-3-R. For 5'-RACE, the 5'-sense primer included in the 5'-RACE kit and GhFT-5-F and GhFT-5-R were used for inverse primers to isolate the 5'-end of GhFT1. GhFT-F2 and GhFT1-R2 were used to obtain the ORF and construct plant expression vectors. gGhFT-F and gGhFT-R were used to obtain the genomic DNA sequences of the GhFT1 using leaf DNA as the template. Primers sequences used in this study are listed in Table S1 online.

### Gene expression analysis

Total RNA was isolated using the Biospin Plant Total RNA Extraction Kit (Bioer, Hangzhou, China) according to the manufacturer's protocol. The cDNA synthesis reactions were

performed using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with 1 µg of total RNA per reaction used as template. Quantitative RT-PCR was carried out using Applied Biosystems 7500 Fast Real-Time PCR System and Fast SYBR Green Master Mix (Life Technologies, Foster City, CA, USA) to detect *GhFT1* gene expression patterns in different tissues and stages during fiber development, and daily oscillation, respectively. The gene-specific primers were qRT-F and qRT-R for *GhFT1*, and UBQ7-F and UBQ7-R for cotton *Ubiquitin7* (GenBank accession no. DQ116441) which was used as an internal control. Gene-specific primers AtAP1-F and AtAP1-R, AtSOC1-F and AtSOC1-R, AtLFY-F and AtLFY-R, and AtFUL-F and AtFUL-R that were designed based on a previously published paper (Zhang et al. 2013) were used to detect AtAP1, AtSOC1, AtLFY, and AtFUL in transgenic *Arabidopsis* and WT plants, respectively. Actin-F and actin-R were used to amplify the *AtActin2* gene (GenBank accession no. NM\_180280) which was used as an internal control. At least three replicate assays were performed with independently isolated RNA for all experiments. Each RT reaction was loaded in triplicate for qRT-PCR analysis. Quantitative RT-PCR data were analyzed using the PCR analysis program 7500 software version 2.0. (Life Technologies, Foster City, CA, USA)

#### Phylogenetic analysis

To generate a phylogenetic tree, *GhFT1* and FT proteins from other plant species were aligned with Clustal W using the default parameters. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al. 2013) (neighbor-joining, Poisson correction distance model). Bootstrap analysis was performed to estimate nodal support on the basis of resamplings.

#### Transformation of *Arabidopsis thaliana*

*Agrobacterium tumefaciens* GV3101 (pMP90RK), harboring a binary vector, was used to transform *Arabidopsis* accession Col-0, ft-10 mutant plants using the floral dip method (Clough and Bent 1998). 35S::*GhFT1* was constructed by introducing *GhFT1* coding sequences downstream of the CaMV 35 S promoter of the binary vector pCAMBIA 2300-35S-OCS (Hajdukiewicz et al. 1994). Transgenic plants were selected on half-strength MS culture medium containing 50 µg/mL kanamycin. Homozygotes were replanted and subsequently monitored for flowering using non-transgenic WT seedling as control. Flowering time was measured as the rosette number of leaves per plant.

#### Subcellular localization analysis

For the green fluorescent protein *GhFT1*-GFP fusion protein construct (with GFP at the C-terminus of *GhFT1*), the 0.7 kb GFP coding sequence was first ligated into the *Xba*I and *Sall* sites of the binary vector of pCAMBIA2300-35S-OCS to construct 35S::GFP. The coding regions of *GhFT1* without stop codon were amplified by PCR and inserted into the *Kpn*I and *Xba*I sites of the 35S::GFP vector to generate 35S::*GhFT1*-GFP in-frame fusion. The fusion construction was then introduced into *Arabidopsis* accession Col-0 by *A. tumefaciens* (GV3101). The selected T1 transformants were used to detect GFP fluorescence by confocal microscope (LSM510; Zeiss, Jena, Germany).

## ACKNOWLEDGEMENTS

We are grateful to Professor Xiangdong Fu, The State Key of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences, for the critical comments on the manuscripts, and for providing us with the pCAMBIA2300-35S-OCS vector. This work was supported by the National Natural Science Foundation of China (31360366), the Program for New Century Excellent Talents in University (NCET-12-1072), and the Doctor Science Foundation of Xinjiang Production and Construction Corps (2012BB007).

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

**Figure S1.** Schematic diagram of gene structures and sequence alignment identification of FT homologs in cotton

**Figure S2.** Phylogenetic analysis of cotton FT and TFL1 homologs with a representative subset of 49 amino acid sequences belonging to the PEBP family (TFL1, FT, MFT-like)

**Figure S3.** Green fluorescence protein (GFP) image of root tip cells in 35S::GhFT1-GFP transgenic plant

**Figure S4.** Flowering phenotype of transgenic *arabidopsis* line expressing GhFT1

**Figure S5.** Expression level of GhFT1 gene in wild-type *Arabidopsis* (Col-0) and transgenic line 5 and 12 expressing GhFT1

**Table S1.** Sequences of the primers used in this study

**Table S2.** Transgenic expression of FT-like genes in *arabidopsis*

**Table S3.** Percentage identity (horizontal) and divergence (vertical) of proteins among GhFT1 and 23 FT orthologs which can promote precocious flowering when ectopically overexpressed in *Arabidopsis thaliana*