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Identification of Members of the Dimocarpus Longan Flowering Locus T Gene Family with Divergent Functions in Flowering

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Abstract *Dimocarpus longan* is a subtropical fruit crop whose year-round production relies on the application of $KClO_3$ to induce flowering; however, the mechanism by which this chemical causes flowering is yet unknown. To further characterize floral signaling in this species, we have isolated three longan *FLOWERING LOCUS T (FT)*-like genes and studied their activities by heterologous expression in Arabidopsis. Expression of two of these genes (*DlFT2* and *DlFT3*) accelerates flowering, whereas expression of the third gene (*DlFT1*) causes delayed flowering and produced floral morphology defects. This anti-florigenic protein may be a member of a class of *FT*-like family involved in flowering time control in biennial and perennial species. Surprisingly, $KClO_3$ treatment also suppressed the expression of both *DlFT2* and *DlFT3* in a field trial.

Keywords FT · Flower induction · Potassium chlorate · Antagonistic function

Abbreviations

FT	Flowering locus T
RACE	Rapid amplification of cDNA ends
BAC	Bacterial artificial chromosome
TFL1	Terminal flower 1
DTB	Days to bolting

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Introduction

Longan (*Dimocarpus longan*), a subtropical fruit crop in the Sapindaceae family, exhibits unique floral induction in response to a soil application of potassium chlorate (Yen 2000), and related chemicals (Matsumoto et al. 2007). The mechanism underlying this response remains elusive and this is confounded by the toxicity of such compounds to other eukaryotes. Chlorate induced flowering of longan allows off-season and year-round fruit production, a capability not exhibited by other crops. Discovery of the molecular mechanism underlying this type of floral induction has enormous potential for the development of new tropical and temperate fruit cultivars by incorporating novel flowering triggers.

Only a few studies on longan reproductive development have been reported, but comprehensive genomic sequencing of this species has not been done. A previous report (Matsumoto 2006) used suppressive subtractive hybridization PCR to differentially amplify transcripts expressed in vegetative and floral bud tissues of $KClO_3$-treated trees. Whereas this approach did identify a number of differentially expressed transcripts, a major limitation to this analysis was that the phenotype (floral bud development) is undetectable for 7 weeks post-application; thus, the identified transcripts are much more likely to be involved in conferring meristem identity than to be involved in signal detection. The uncertain lag duration from soil $KClO_3$ application to effecting a change in gene expression that ultimately leads to floral transition in buds is a major complication in characterizing chlorate induced flowering at the molecular level.

FLOWERING LOCUS T (FT) has been identified as the key integrator of floral transition stimuli in vascular plants and is a phloem-mobile protein hormone expressed in leaves and translocated to meristems (reviewed in (Zeevaart 2008)). Signaling through FT protein is thought to be universal in angiosperms and FT genes have been identified from a number of crops, including grapevine, apple, citrus, pineapple, sugar beet and poplar (Carmona et al. 2007; Hsu et al. 2006; Kobayashi et al. 1999; Kotoda et al. 2010; Lv et al. 2012; Pin et al. 2010). Upon translocation to meristems, FT associates with the Basic Leucine Zipper (bZIP) transcription factor FD (FLOWERING LOCUS D) and forms activation complexes driving the expression of floral identity genes including *APETALA1* (*AP1*) (Abe et al. 2005; Wigge et al. 2005). TERMINAL FLOWER 1 (TFL1), a related protein with 54 % sequence identity to FT also binds FD, but the formation of such complexes results in transcriptional repression of *AP1* (Wigge et al. 2005). FT and TFL1 are members of a six member protein family in Arabidopsis similar to mammalian phosphatidylethanolamine-protein (Schoentgen et al. 1987), also including TWIN SISTER OF FT (TSF), BROTHER OF FT (BFT), *Arabidopsis thaliana* CENTRORADIALIS homolog (ATC/AtCEN), and MOTHER OF FT AND TFL1 (MFT) (Bradley et al. 1997; Kardailsky et al. 1999; Kobayashi et al. 1999; Mimida et al. 2001; Yoo et al. 2010, 2004). Identity comparisons of each of the Arabidopsis FT/TFL1 proteins, as well as those characterized from horticultural crops are shown in Table 1.

Involvement of a leaf derived signal in sensing floral cues in longan, as well as lychee and mango, is supported by defoliation and girdling experiments (Davenport et al. 2006; Ramırez et al. 2010; Tiyayon et al. 2010; Ying and Davenport 2004), indicating one or more FT-like proteins are likely involved in floral signaling in the Sapindaceae. In order to identify the genes encoding FT-like proteins of longan, we cloned full-length transcripts by RACE (rapid amplification of cDNA ends) and surveyed a genomic BAC library to identify genomic DNA sequences encoding three distinct FT-like proteins and functionally analyzed these genes by heterologous expression in Arabidopsis.

Results

Cloning and Sequence Analysis of FT-like Genes in Longan

Degenerate primers were used to amplify three partially overlapping longan *FT* cDNA fragments. Full-length cDNA sequences for three different *DlFT* genes were amplified through 5′ and 3′ RACE using internal primers derived from the partial sequences. The three genes were determined to be

Table 1 Percent identity of each protein in Fig. 3 to the Arabidopsis FT/TFL family proteins

	AtFT	AtTSF	AtTFL1	AtBFT	AtCEN	AtMFT	Accession[a]	Reference
AtFT	–	81 %	54 %	55 %	54 %	45 %	AT1G65480	(Kardailsky et al. 1999)
AtTSF	81 %	–	52 %	52 %	54 %	47 %	AT4G20370	(Yamaguchi et al. 2005)
PtFT1	76 %	74 %	55 %	58 %	54 %	50 %	POPTR_0008s07730.1	(Hsu et al. 2011)
VvFT	78 %	75 %	55 %	61 %	53 %	51 %	ABI99465	(Carmona et al. 2007)
BvFT2	75 %	76 %	56 %	55 %	56 %	54 %	ADM92609	(Pin et al. 2010)
MdFT1	72 %	73 %	54 %	54 %	53 %	52 %	BAD08340	(Kotoda et al. 2010)
CiFT	72 %	71 %	56 %	58 %	54 %	49 %	BAA77836	(Kobayashi et al. 1999)
AcFT	69 %	69 %	50 %	56 %	51 %	49 %	ADU15498	(Lv et al. 2012)
DlFT2	70 %	69 %	53 %	54 %	50 %	51 %	KF881010	This Report
DlFT3	70 %	70 %	53 %	54 %	50 %	52 %	KF881011	This Report
DlFT1	69 %	68 %	53 %	54 %	50 %	50 %	KF881012	This Report
BvFT1	69 %	69 %	50 %	56 %	51 %	46 %	ADM92607	(Pin et al. 2010)
PtFT2	72 %	73 %	56 %	58 %	53 %	50 %	POPTR_0010s18680.1	(Hsu et al. 2011)
AtTFL1	54 %	52 %	–	56 %	65 %	47 %	AT5G03840	(Bradley et al. 1997)
VvTFL1	55 %	52 %	67 %	60 %	78 %	49 %	ABI99466	(Carmona et al. 2007)
MdTFL1	54 %	55 %	72 %	61 %	72 %	53 %	BAD06418	(Kotoda et al. 2010)
CsTFL1	56 %	55 %	72 %	62 %	73 %	51 %	AAR04683	(Pillitteri et al. 2004)
PnTFL1	55 %	56 %	69 %	58 %	76 %	50 %	BAD22599	(Igasaki et al. 2008)
AtBFT	55 %	52 %	56 %	–	56 %	48 %	AT5G62040	(Yoo et al. 2010)
AtCEN	54 %	54 %	65 %	56 %	–	50 %	AT2G27550	(Mimida et al. 2001)
AtMFT	45 %	47 %	47 %	48 %	50 %	–	AT1G18100	(Yoo et al. 2004)

[a] Accession numbers indicate GenBank records, TAIR10 records (Arabidopsis genes), or PopGenIE v2.2 records (PtFT1 and PtFT2)

distinct by using gene specific primers within unique regions of the 5′ and 3′ UTRs. DNA hybridization analysis of genomic DNA and BAC clone DNA (Fig. 1) revealed the presence of a fourth *DlFT* gene, *DlFT4*; however, the sequence of *DlFT4*, when compared to *DlFT3*, has only two silent polymorphisms within the CDS region, hence both encoded proteins are identical. GenBank accession numbers for *DlFT1*, *DlFT2 and DlFT3* are shown in Table 1, and the accession number for *DlFT4* is KF881013. The full-length DNA sequence alignment is shown in Supplemental Fig. S-1. Exons 1 through 4 of each of the four genes contain 203, 60, 39, and 223 nucleotides, respectively. *DlFT1* and *DlFT2* are the most divergent, sharing only 88 % sequence identity, whereas the remainder of the pairwise identity comparison values for the four *DlFT* genes are 90 % to 99 % identical.

Functional Analysis of FT-like Genes

To evaluate the floral induction potential of each of the longan FT proteins, we utilized heterologous expression in Arabidopsis. Overexpression constructs for each *DlFT1*, *DlFT2*, *DlFT3*, as well as Arabidopsis *FT* were introduced into wild-type (Ler-0) and *ft-3* mutant backgrounds. The *ft-3* mutant carries the mutation R119H (Arg^{119}→His), and has a 16 day flowering time delay relative to the wild-type plants (Kardailsky et al. 1999). The effect on flowering time, measured in number of days to bolting (DTB), for each construct is shown in Table 2. As expected, the 35S::*AtFT* construct dramatically reduced flowering time; within one segregating T2 population (35S::AtFT_T2_12), DTB was significantly reduced by an average of 13.58 days for individuals having the allele relative to their wild-type siblings. Overexpression of *DlFT2* and *DlFT3* likewise reduced flowering time in the *ft-3* background, and stable T3 homozygous lines were generated having average DTB reductions 5.84 and 9.51 days, respectively.

In T1 generation plants, overexpression of *DlFT1* in the *ft-3* background caused delayed flowering and a floral morphology phenotype wherein inflorescences developed clusters of leaves instead of flowers (Fig. 2a and d). These plants did eventually flower and produce viable seed, but the morphological defect was not observed in subsequent generations, nor were there significant differences in DTB between homozygous *DlFT1* T3 populations and azygous *ft-3* populations (Table 2). No detectable reduction in DTB was observed when *DlFT2* or *DlFT3* were introduced into wild-type Ler-0 plants (data not shown). However, overexpression of *DlFT1* in the wild-type Ler-0 background caused delayed floral development (Fig. 2b and e), although the phenotype was less pronounced than in the *ft-3* backgound and DTB was unaffected.

Previous reports demonstrated that floral inducing and retarding activities can be exchanged between FT/TFL1 family member proteins by swapping domains or even single

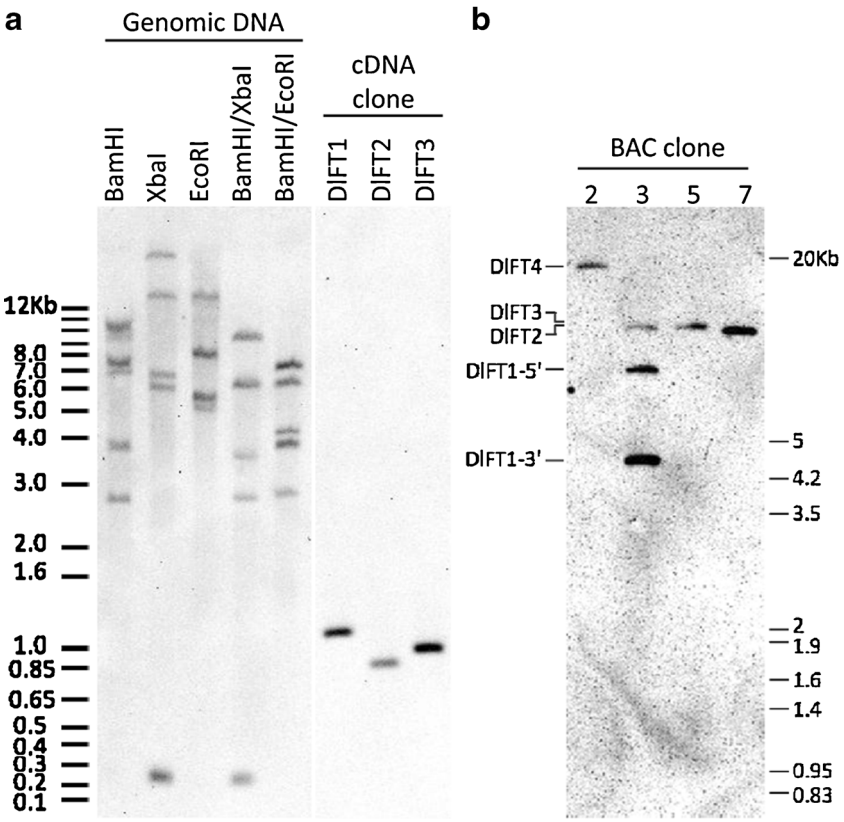

Fig. 1 Southern blot analysis of longan *FT* genes. **a** Longan genomic DNA digested with indicated restriction enzymes or plasmids containing DlFT cDNA clones digested with EcoRI and probed with a fragment of *DlFT1* exon 4. **b** BAC DNA digested with EcoRI probed with full-length *DlFT1* cDNA

Table 2 Effect of FT gene overexpression on flowering time in Arabidopsis

Construct	Generation	Line ID	Genotype	No. plants	Avg. DTB	SD. DTB
35S::AtFT	T2	12	PCR+	16	16.00[a]	0
			PCR-	12	29.58	1.44
	T2	14	PCR+	19	18.15[a]	2.75
			PCR-	9	29.44	2.24
	T2	11	PCR+	10	26.30[b]	3.53
			PCR-	4	31.50	1.14
35S::DlFT3	T2	2	PCR+	22	22.41[a]	1.29
			PCR-	6	31.16	1.09
	T3	2_11	Homozygous	19	22.74[a]	1.52
		2_1	Azgous	20	32.25	1.91
	T2	4	PCR+	11	28.64	1.57
			PCR-	3	31.00	1.73
	T2	1	PCR+	25	21.60	0.96
			PCR-	2	30.00	1.41
35S::DlFT2	T3	2_11	Homozygous	18	27.22[a]	0.65
		2_15	Azgous	18	33.06	3.15
	T2	8	PCR+	8	27.63[a]	1.06
			PCR-	6	31.83	2.14
35S::DlFT1	T3	7_101	Homozygous	18	32.11	0.47
		7_104	Azgous	18	32.56	1.39
	T3	8_102	Homozygous	20	33.50	3.76
		8_103	Azgous	19	32.78	2.32

Genetic background used is *ft-3* mutant. DTB, days to bolting. Statistically significant changes in DTB between genotypes indicated by superscripts: [a] $P \leq 0.01$, [b] $P \leq 0.05$; P-value calculated using one-tailed Student's T-test

amino acids between the molecules (Ahn et al. 2006; Hanzawa et al. 2005; Pin et al. 2010). In a swapping experiment, segment B within the fourth exon of the encoded Arabidopsis FT and TFL1 proteins was identified as causing the functional divergence between the two molecules (Ahn et al. 2006). Figure 3 shows an alignment of the protein sequences encoded by the fourth exon of the six member Arabidopsis FT/TFL1 family, the three longan FT proteins, as well as several additional FT and TFL1 proteins characterized from horticultural crops. Full-length protein alignments are shown in Supplemental Fig. S-2, and identity analysis of the Arabidopsis FT/TFL1 protein family and a comparison of each of the crop species FT proteins to the Arabidopsis proteins is shown in Table 1. To investigate the functional polymorphisms between DlFT1 and DlFT2/DlFT3 causing their opposing effects on floral development, we generated mutated alleles of DlFT1 and DlFT3 by swapping particular amino acids of the encoded proteins to that of the opposite molecule. Residues of Asn^{133} and Lys^{142} of DlFT1, corresponding to Tyr^{133} and Asn^{142} in DlFT3, as well as a set of three residues in the carboxy-terminius segment D were selected for the mutation analysis. Phenotypic analysis of T1 plants carrying longan FT alleles with single mutations or combinations thereof are shown in Table 3. None of the mutations to DlFT1 changed this gene to a floral inducer; however, the mutation DlFT3 Y133N singly or in combination with N142K caused the leafy flower phenotype associated with DlFT1, indicating that Tyr^{133} is a critical residue for flower induction.

Gene Expression Analysis of FT-like Genes Under KClO3 Treatment

We also sought to determine if the expression of the longan *FT* genes is influenced (i.e., up-regulated) by chemical induced flowering in trees treated with potassium chlorate. Quantitative real-time PCR was used to detect the expression level of *FT* transcripts in mature leaves. Expression in other tissues including tissue from various stages of bud development and roots were not detectable. *FT* transcript abundance relative to *ACTIN* and *EF1α* showed sharp and approximately equivalent down-regulation of all three *FT* transcripts in response to chlorate application, as shown in Fig. 4. By the 16th day post-application, a

Fig. 2 DlFT1 overexpression phenotype. **a** and **d** Two independent T1 transformants expressing 35S::*DlFT1* in the *ft-3* mutant background displaying the leaf-like flowers phenotype. **b** and **e** two views of a T2 generation Ler-0 background 35S::DlFT1 overexpressing plant; a wild-type sibling is shown in (**c**) and (**f**). Note the delayed floral development in (**b**) and (**e**) compared to (**c**) and (**f**). Ages of plants in **a** and **d** are 52 days, and 38 days in (**b**), (**c**), (**e**) and (**f**)

reduction of approximately 100-fold for each transcript was observed relative to the day 0 measurements in two biological replicates, whereas expression in the untreated control trees did not fluctuate more than 3-fold for any of these transcripts. Beyond day 16 post-application, we were unable to detect expression of *FT* transcripts at weekly sampling intervals leading up to the emergence of flowers that occurred 7 weeks after chlorate application.

Discussion

In the absence of whole-genome and transcriptome data for a horticultural crop, characterization of FT-like proteins is a valuable entry point for characterization of floral signaling. Because FT acts as a universal integrator of signals from various stimuli, we predicted that one or more FT-like proteins are involved in vegetative to floral transition in longan. After identifying the longan family of *FT* genes by cDNA amplification and enumerating the family members by DNA hybridization, we functionally tested each of the three different proteins for floral inducing activity by heterologously expressing them in Arabidopsis, as has been done for other horticultural species (Carmona et al. 2007; Hsu et al. 2011; Kotoda et al. 2010; Lv et al. 2012; Pin et al. 2010).

The overall level of sequence homology between the three longan FT proteins to Arabidopsis FT is nearly equal (70 % for DlFT2 and DlFT3, and 69 % for DlFT1), and all three proteins share only 53 % sequence identity with Arabidopsis TFL1 (Table 1). This observation indicates that DlFT1 is not a longan ortholog of Arabidopsis TFL1, but instead is a member of a poorly understood class of FT-like proteins with a divergent function that is beginning to be identified in biennial and perennial species. Expression of 35S::*DlFT1* produces floral delay phenotypes associated with *TFL1* genes (Ahn et al. 2006; Hanzawa et al. 2005; Kobayashi et al. 1999), and produces floral development defects similar to those observed with overexpression of grapevine *VvTFL1A* (Carmona et al. 2007).

FT/TFL1 family proteins can be separated into four groups on the basis of sequence and function (Fig. 3 and Supplemental Fig. S-2). Class I contains FT and TSF, which is FT-like in sequence and induce flowering (Kardailsky et al. 1999;

Yamaguchi et al. 2005); class II proteins are similar to FT in sequence but inhibit flowering; class III contains TFL1 and two additional Arabidopsis proteins, AtBFT and AtCEN, which have similar sequences and functionally inhibit flowering (Bradley et al. 1997; Mimida et al. 2001; Yoo et al. 2010); and the final class, IV, contains Arabidopsis MFT, which by overexpression accelerates flowering but loss-of-function allele, *mft-1*, has no effect (Yoo et al. 2004). DlFT2 and DlFT3 clearly belong to class I as heterologous expression expedites flowering in the Arabidopsis system (Table 2), and DlFT1, belongs to a class II based on its floral repression phenotype (Fig. 2).

Analysis of FT protein structure and function benefits from both an x-ray crystal structure (Ahn et al. 2006) and mutation analysis of AtFT and AtTFL whereby each was modulated to encode opposite functions by changing single amino acids (Hanzawa et al. 2005) or larger protein segments (Ahn et al. 2006). Tyr^{88} of AtFT, a key residue differentiating FT from TFL1, (Hanzawa et al. 2005) is conserved in all three DlFT proteins (Supplemental Fig. S-2); however, several differences exist between DlFT1 and DlFT2/DlFT3 within the segments encoded by the fourth exon, which was also shown to contain functional differences between AtFT and AtTFL1 (Ahn et al. 2006). Specifically, segment B of exon 4 was shown to be necessary and sufficient to interchange AtFT and AtTFL1 functions (Ahn et al. 2006), and within the corresponding aligned region of the longan proteins, DlFT1 Asn^{133} is the only change from an absolutely conserved residue in class I FT proteins. Another candidate residue of the DlFT1 selected for mutation analysis included Lys^{142}, as this residue confers a charge difference from the corresponding conserved asparagine residue in DlFT2/DlFT3 and other class I FT proteins. Lastly, a set of residues near the C-terminus of DlFT1, Ile^{167}, Ile^{168}, and Ser^{172}, were tested for functional significance as these amino acids are not conserved in class I proteins, nor in any of the other classes. Mutation of DlFT3 Y133N caused a leafy flower phenotype associated with DlFT1 overexpression; whereas none of the mutant alleles of DlFT1 caused accelerated flowering phenotypes associated with DlFT3. Another possible residue of DlFT1 that may alone, or in combination with other residue(s) (e.g. N133) prevent formation of activation complexes is Gln^{127}. Like Asn^{133}, Gln^{127} lies within segment B, and likely causes a very different topology of the external loop of DlFT1 from DlFT2/DlFT3 in which both proteins have a proline residue at this position.

One possible role of class II FT proteins may be differentiation between juvenile and reproductively competent developmental stages in plants requiring multiple growing seasons to reproduce. The two other class II proteins, BvFT1 from sugar-beet (a biennial) (Pin et al. 2010) and perennial poplar PtFT2 (Hsu et al. 2011), are divergent from their class I homologs in the critical B segment, which was functionally tested in the case of BvFT1 (Klintenas et al. 2012; Pin et al. 2010) and proposed in poplar (Hsu et al. 2011). Therefore, it is not surprising that the Asn^{133} of DlFT1, which is within the B segment, likewise determines its role as a floral inhibitor.

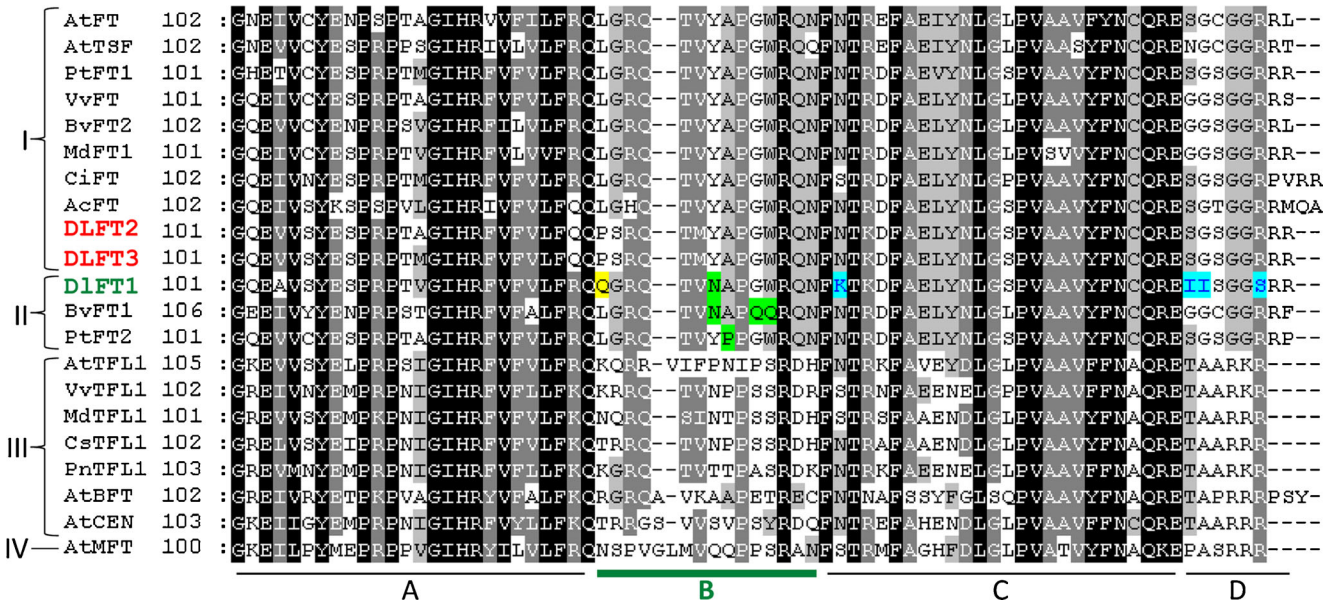

Fig. 3 Alignment of the FT/TFL protein family from Arabidopsis with characterized FT and TFL1 genes from crop species. *Arabic numbers* indicate residue number. *Roman numerals* indicate FT/TFL protein sequence and function class. **a**–**d** refer to previously defined segments of AtFT and AtTFL1 fourth exons used in (Ahn et al. 2006). Key residues distinguishing TFL1 function from FT function are indicated by green highlighting (Ahn et al. 2006; Hsu et al. 2011; Pin et al. 2010). *Blue highlighting* indicates residues of DlFT1 that when substituted into a DlFT3 expression construct did not cause a functional change. *Yellow highlighting* indicates an additional residue of DlFT1, Gln^{127}, not yet tested for functional significance. See Supplemental Fig. S-2 for the full-length sequence alignment, and Table 1 for sequence accession numbers

Table 3 Effect of mutations in overexpression constructs on Arabidopsis flowering

	Mutation	Effect
35S::DlFT1	N133Y	No change
	K142N	No change
	K142R	No change
	N133Y and K142N	No change
	ΔC-terminus DlFT3	No change
35S::DlFT3	Y133N	Leafy Flowers
	N142K	No change
	Y133N and N142K	Leafy Flowers
	ΔC-terminus DlFT1	No change

The antagonistic effects between FT and TFL1 are thought to be caused by competition for binding common a transcription factor, FD, which is capable of forming both activation and repression complexes, depending on whether FT or TFL1 proteins are bound (Abe et al. 2005; Wigge et al. 2005). The topology of the FT/TFL1 molecule, as determined by the exposed residues external loop (segment B) identified in (Ahn et al. 2006) or required for secondary structure formation, e.g. the histidine-tyrosine polymorphism between FT-TFL1 characterized in Hanzawa et al. (2005), likely determines what additional factors bind the FT/FD or FT/TFL1 transcriptional activation or repression complexes. Therefore, the same mechanism directing the antagonism between class I and class II FT proteins can be postulated. Aside from the formation of genuine transcriptional activation complexes, individual FT/TFL1 proteins can sequester common factor(s) (e.g. FD) thereby preventing the association of this protein into functional complexes, which is one outcome believed to cause phenotypes in chimeric FT/TFL1 proteins or other mutants of these proteins (Ahn et al. 2006). Dominant negative interference of FT paralogs has been observed in domesticated sunflower, in which photoperiod specific expression of a frameshift mutated product, HaFT1-D, interferes with the floral stimulating activity of HaFT4 (Blackman et al. 2010) impeding floral activation under long day conditions. Indeed, floral signaling mediated through FT/TFL1 family proteins is not likely dependent on the absolute expression level of a single protein, but is a result of the complex spatiotemporal expression

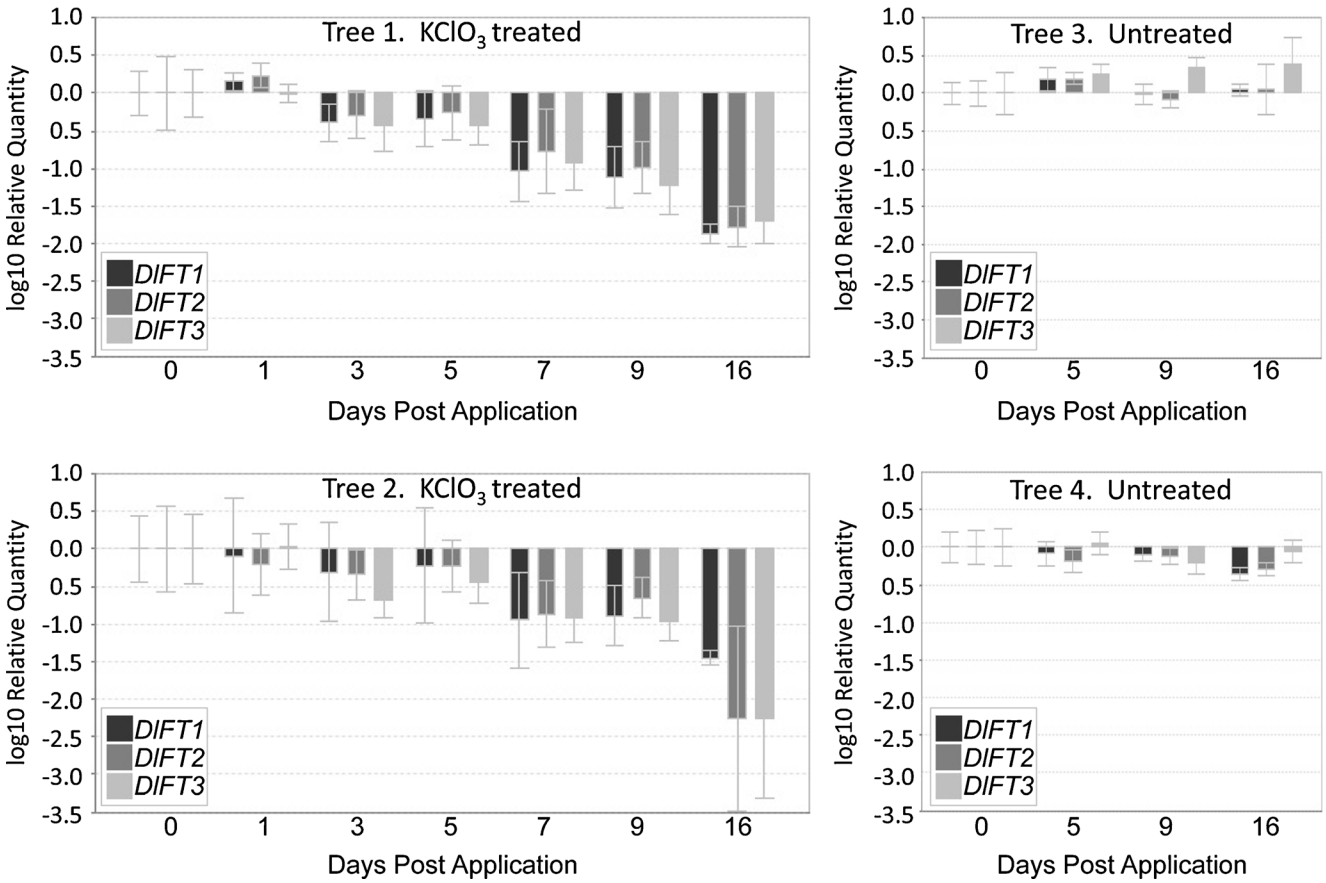

Fig. 4 FT gene expression is down-regulated following application of $KClO_3$. Change in transcript abundance is shown as the log10 quantity of transcripts on days indicated relative to their abundance at day 0. Transcript abundance was calculated using the $\Delta\Delta C_T$ method using *ACTIN* and *EF1α* multiple endogenous controls and day 0 sample from each tree as reference. Three technical replicates were performed per sample and error bars were calculated as a pooled combination of the individual variability estimates

patterns of multiple antagonistic factors and their abundance relative to one another.

Our results did not show a positive correlation between expression of any *DlFT* gene and potassium chlorate inducted flowering of longan. Given that many FT/TFL1 family proteins are unknown and uncharacterized in longan, it is impossible to know the relative abundance of activation or repressor proteins is chemically induced trees or in untreated trees experiencing non-inductive or naturally inductive conditions. It is possible that chlorate treatment could modulate the relative abundance of FT/TFL1 proteins through differences in protein stability and turnover, independent of transcript abundance. Because FT/TFL1 proteins compete for binding partners, the overall activity of the complexes they form can be shifted by modest changes in protein abundance. Following a generalized down-regulation of transcription affecting all members to a similar degree, the ratio can be of florigenic to antiflorigenic activities could altered by one or more of the FT proteins persisting longer than others. Therefore, it is not necessary for protein stability to make up for the 100-fold change in absolute transcript abundance, such as we observed in chlorate treated trees, but a change as small as 2-fold in protein stability could realistically shift the steady state level of florigenic complexes.

Overall, our experiments have identified two FT-like proteins that are positive regulators of flowering (DlFT2 and DlFT3) and one negative regulator of flowering (DlFT1). The genes encoding these antagonistic proteins are appealing targets for characterization in cultivars with varying inductive requirements (e.g. SNP analysis, gene expression analysis). Additionally, characterization of factors acting upstream of the *FT* genes or their *cis*-regulatory elements represent additional avenues for identification and design novel floral induction mechanisms for use in longan or other crops.

Methods

Oligonucleotides

A list of primers (Eurofins MWG Operon, Huntsville, AL) used for these experiments is shown in Supplemental Table S-1.

Plant Materials and Growth Conditions

Dimocarpus Longan 'Biew Kew' longan trees grown at the University of Hawaii, Waiakea Agriculture Research Station, Hilo, Hawaii were used for this study. $KClO_3$ treatment consisted of a granular application of 250 g/tree $KClO_3$ under the tree canopy followed by irrigation. For the RT-qPCR study, two trees were treated on 4 September, 2012; control samples were collected from untreated trees.

Arabidopsis Thaliana Arabidopsis Ler0 (wild-type) and *ft-3*, stock CS184, were obtained from the ABRC (Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH). Plants were grown in chambers under 16 h photoperiod with 75 % relative humidity. Individual plants were observed for floral development phenotypes.

Cloning of the Longan *FT* Gene Family

Partial cDNA Isolation Using Degenerate Primers First strand cDNA was synthesized from total leaf RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) primer following the manufacturer's instructions. Three primer combinations were used for RT-PCR with Platinum Taq (Invitrogen): Degen_FT_1F/Degen_FT_1R, Degen_FT_2F/Degen_FT_2R, Degen_FT_3F/Degen_FT_2R to amplify fragments of 349, 230, and 167 bp, respectively.

RACE (Rapid Amplification of cDNA Ends) Poly(A) RNA (200 ng) was isolated from 400 mg leaf tissue using the RNaqueous and MicroPoly(A)Purist kits (Ambion, Austin, TX). This was used as the input for the Generacer RLM-RACE kit (Invitrogen, Carlsbad, CA) cDNA synthesis following the manufacturer's protocol. RACE primers were designed to overlap variable regions within the existing partial cDNA sequences. 5′ RACE products for *DlFT1* and *DlFT2* were both isolated using primer DlFT1/2_R and GR5′O and differentiated by sequence analysis. The 5′ RACE product of *DlFT3* was produced by nested PCR using the DlFT3_+367R primer with GR5′O primer for the outer reaction and with GR5′N for the nested reaction. 3′ RACE products were produced by nested PCR using primers DlFT1_Exon4_F, DlFT2_+310F, and DlFT3_+367R first with GR3′O and subsequently with GR3′N, for DlFT1, DlFT2 and DlFT3, respectively. Full length *DlFT1*, *DlFT2*, and *DlFT3* transcripts were amplified from the Generacer RT template using primer combinations DlFT1_-87F/DlFT1_+806R, DlFT2_-78F/DlFT2_+600R, and DlFT3-78F/DlFT3_+675R, respectively. GoTaq polymerase (Promega, Madison, WI) was used for initial RACE reactions, and Phusion DNA polymerase (Thermo Fisher, Waltham, MA) was used for full-length transcript amplification. All products were TOPO cloned into pCR4/TOPO (Life Technologies) following PCR clean-up (QIAquick PCR Purification kit, Qiagen, Valencia, CA). In the case of Phusion PCR products, A-tailing was performed by adding 1 μl GoTaq to Phusion reactions and incubating at 72 °C for 5 min prior to PCR cleanup.

Southern Blot Analysis Genomic DNA extraction was by the Nucleospin Plant II Maxi kit (Machelry Nagel, Bethlehem, PA) using extraction buffer PL1 on leaf tissue and the

manufacturer's protocol. Restriction enzymes *Xba*I, *Bam*HI and *Eco*RI were selected as their recognition sequences were absent from the fourth exon of each cDNA sequence. Digested DNA, 35 μg/lane was blotted onto Hybond-N+(GE Healthcare, Piscataway, NJ). A DIG labeled *DlFT1* exon 4 probe was prepared for hybridization using the PCR DIG Labeling Kit (Roche, Indianapolis, IN) and primers DlFT1_Exon4_F and DlFT1_+525R. Following overnight hybridization in DIG EasyHyb (Roche) at 42 °C, the blot was washed, blocked, probed and developed using the DIG Wash and Block Buffer Set, Anti-Digoxigenin-AP Fab fragments, and CDP-Star according to the manufacturer's protocols (Roche).

BAC Library Analysis A 55,296-clone longan BAC DNA library was prepared by Amplicon Express (Pullman, WA). A full-length DlFT1 DIG probe (DlFT1_-87F/DlFT1_+525R) was used to probe the library filters and identify DlFT gene containing clones. Small scale BAC DNA preparations (PerfectPrep BAC 96 kit, Eppendorf, Hauppauge, NY) digested with EcoRI were used for a Southern blot to identify unique restriction fragment hybridization patterns. Large scale BAC preps (Large Construct Kit, Qiagen), were used to prepare DNA for direct sequencing.

Bioinformatic Analyses

Databases queried by BLAST to obtain sequence data included NCBI, TAIR 10 (www.Arabidopsis.org), and TIGR Plant Transcript Assemblies (Childs et al. 2007). Alignment of nucleotide and protein translations was by T-COFFEE (Notredame et al. 2000) and files were viewed and edited with GeneDoc (http://www.psc.edu/biomed/genedoc). DNA sequencing of PCR products, plasmids and BACs was by Eurofins MWG Operon (Huntsville, AL).

Heterologous Expression of DlFT Genes in Arabidopsis

In order to test the ability of the longan FT genes to complement the flowering delay phenotype in line *ft-3*, the coding sequence of each was amplified using primers with overhanging restriction sites. XhoI-DlFT1-ApaI, XhoI-DlFT2-ApaI, XhoI-DlFT3-SalI were amplified by Phusion DNA polymerase using the full-length cDNA clones described above and primers listed in Supplemental Table S-1; likewise XhoI-AtFT-ApaI was amplified from cDNA clone G11796 (ABRC). The XhoI-ApaI fragment from the *DlFT1*, *DlFT2* and *AtFT* clones and the XhoI-SalI fragment from the DlFT3 clones were introduced into the 35S expression cassette of pEPJ86. Subsequently the expression cassettes were subcloned into pBINPLUS/ARS (Belknap et al. 2008). Arabidopsis transformation was done by the floral dip method into wild-type Ler-0 and *ft-3* mutant backgrounds (Clough and Bent 1998) using *Agrobacterium tumifaciens* GV3101 and the selection of primary transformants was in MS agar containing 50 μg/ml kanamycin and 100 μg/ml carbenicillin.

Mutant alleles of *DlFT1* and *DlFT3* containing one or more codons changed to that of the opposite gene were synthesized by producing overlapping PCR products with internal sequence alterations, then fusing the two products together by overlap PCR using the 5′ or 3′ end primers. Mutant alleles were transformed only into the *ft-3* mutant background. All alleles were confirmed by sequencing of two complementary strands prior to transformation into Arabidopsis (Eurofins MWG Operon).

Quantitative Real-Time RT-PCR

Endogenous Control Primer Design ACTIN and *EF1α* have been identified by previous studies to be superior reference genes for RT-qPCR analysis in longan and lychee (Lin and Lai 2010; Zhong et al. 2011). However, the particular primers used for quantitation of both transcripts by Lin and Lai (2010) were unable to discriminate between genomic DNA and cDNA based on the size of genomic DNA PCR and RT-PCR products. In order to design a new *ACTIN* primer set with this characteristic, the partial cDNA clone (GenBank, EU340557) was queried against Arabidopsis transcripts and At3g12110.1 was identified as the closest homolog. Alignment of the genomic and cDNA sequences for At3g12110.1 was used to predict the exon-intron structure of the longan gene and primers DlACT_+436F and DlACT_+545R were predicted to span the second intron. DNA sequence analysis of PCR/RT-PCR products confirmed this hypothesis and showed these primers produce a 109 bp RT-PCR amplicon and a larger, intron-containing 632 bp genomic product. Similarly, for EF1α, a new primer set was designed using the GenBank accession referenced by Lin and Lai (2010), DQ471426 (*Litchi chinensis*), as well as the following sequences from TIGR plant transcript assemblies: *Citrus sinensis* TA10857_2711, *Poncirus trifoliata* TA5136_37690, and *Citrus clementina* TA2105_85631. Primer EF1α_+1392 was used with GR5′O in a 5′ RACE reaction to amplify a near full-length longan EF1α transcript. A BLAST query against Arabidopsis transcripts identified At1g07920.1 and At1g07940.1 as the closest homologs, whose intron-exon structures was used to predict primers DlEF1α_+427F and DlEF1α_+556R, for which RT-PCR size specificity was confirmed.

DlFT Gene Primers DlFT primers were designed to be specific for a given FT gene target by placement in regions with as much divergence between the target gene and other FT genes as possible. Additionally, these primers have 3′ nucleotides specific to the particular FT target and each primer set crosses 2 introns of genomic sequence allowing discrimination from genomic PCR products. The three primer sets:

DlFT1_+254F/DlFT1_+412R, DlFT2_+221F/DlFT2_+386, and DlFT3_+219F/DlFT3_+404R for DlFT1, DlFT2, and DlFT3, respectively, are depicted on the Supplemental Fig. S-1 sequence alignment. A single FT qPCR probe, DlFT_ALL_PROBE, is designed against a conserved region of the three genes.

Reaction Setup Random hexamer primed cDNA for qPCR was prepared using 500 ng RNA isolated from 200 mg leaf tissue with the RNaqueous and Turbo DNA-free kits (Ambion) and M-MLV Reverse Transcriptase (Promega) according to the manufacturer's protocols. qPCR reactions contained 5 μl SsoFast Probes Supermix (Bio-Rad, Hercules, CA), 900 n*M* primers, 250 n*M* probe, and 1 μl M-MLV RT reaction. Thermal cycling was performed on a StepOne Real-time PCR instrument (Applied Biosystems, Carlsbad, CA) using fast cycling and default parameters and quantitation using the $\Delta\Delta C_T$ method.

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Conflict of Interest The authors declare they have no conflict of interest.

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