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Short communication

Isolation and characterization of a *FLOWERING LOCUS T* homolog from pineapple (*Ananas comosus* (L.) Merr)

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ABSTRACT

FLOWERING LOCUS T (*FT*)-like genes are crucial regulators of flowering in angiosperms. A homolog of *FT*, designated as *AcFT* (GenBank ID: HQ343233), was isolated from pineapple cultivar *Comte de Paris* by reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The cDNA sequence of *AcFT* is 915 bp in length and contains an ORF of 534 bp, which encodes a protein of 177 aa. Molecular weight was 19.9 kDa and isoelectric point was 6.96. The deduced protein sequence of *AcFT* was 84% and 82% identical to homologs encoded by *CgFT* in *Cymbidium goeringii* and *OgFT* in *Oncidium Gower Ramsey* respectively. Quantitative real-time PCR (qRT-PCR) analyses showed that the expression of *AcFT* was high in flesh and none in leaves. qRT-PCR analyses in different stages indicated that the expression of *AcFT* reached the highest level on 40 d after flower inducing, when the multiple fruit and floral organs were forming. The 35S::*AcFT* transgenic *Arabidopsis* plants flowered earlier and had more inflorescences or branches than wild type plants.

1. Introduction

The transition from vegetative growth to flowering is a crucial commitment in plant development. The proper time of flowering ensures successful reproduction and the persistence of populations and species. Studies of flowering time in *Arabidopsis* have proposed four major pathways mediating response to environmental and endogenous cues. Endogenous factors affect the transition to flowering through the autonomous and gibberellin pathways, whereas external signals regulate flowering via the photoperiodic and vernalization pathways (Corbesier and Coupland, 2005; Searle and Coupland, 2004; Yanovsky and Kay, 2003). The signaling pathways receptive to the environmental and endogenous cues are responsible for the accurate regulation of flower induction (Lee et al., 2006). Some studies indicated that *FT* is characterized as a floral pathway integrator (Wigge, 2011). In different plant species, *FT* homologs are involved in the earliest stages of flower development (Zeevaart, 2007).

In *Arabidopsis*, *FT* interacts with the transcription factor *FD* which binds to the promoters of florally expressed genes, such as *AP1*, and ultimately induces flowering (Abe et al., 2005; Wigge et al., 2005). Lately, many *FT* homologs from different species have been isolated and their functions characterized. The *BvFT2* is functionally conserved with *FT* and essential for flowering, in contrast, *BvFT1* represses flowering and its down-regulation is crucial for the vernalization response in beets (Pin et al., 2010). Ectopic over-expression of either *MdFT1* or *MdFT2* in *Arabidopsis* significantly induced early flowering (Li et al., 2010). The transgenic orange plants in which *CiFT* was expressed constitutively showed early flowering, fruiting, and characteristic morphological changes (Endo et al., 2005). Up to now, there is no relevant report of cloning *FT*-like genes from pineapple.

Pineapple is an important herbaceous fruit tree in many tropical and subtropical countries. Natural flowering out of season can cause serious scheduling problems for growers. Flower inducing is a good way to make pineapple flower synchronously, however, some varieties are difficult to induce flowering. There are few report about the mechanism of pineapple flower development. *ACACS2* transgenic pineapple plants exhibiting silencing showed a marked delay in flowering when compared with non-silenced transgenic plants and control non-transformed plants. It is argued that the *ACACS2* gene is one of the key contributors toward triggering 'natural flowering' in mature pineapples (Yuri and José, 2006). *AcPI* was cloned from pineapple and ectopic over-expression of *AcPI* in *Arabidopsis* significantly induced early flowering (Lv et al., 2012). So it is necessary to further

Abbreviations: RACE, rapid amplification of cDNA ends; CaMV, Cauliflower mosaic virus; *FT*, *FLOWERING LOCUS T*; RT-PCR, reverse transcriptase polymerase chain reaction; qRT-PCR, quantitative real-time PCR; *FD*, *FLOWERING LOCUS D*; *AP1*, *APETALA1*; Ct, cycle threshold; ACC, 1-amino-cyclopropane-1-carboxylate; D1, 1 d before flower inducing; D10, 10 d after flower inducing; D20, 20 d after flower inducing; D30, 30 d after flower inducing; D40, 40 d after flower inducing; D50, 50 d after flower inducing.

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know the mechanism of pineapple flower development. In this work, a *FT*-like gene was cloned and its expression analyses were also done.

2. Materials and methods

2.1. Plant materials

Plants of pineapple cultivar *Comte de Paris* were planted in vinyl house. The apical meristems on D1, D10, D20, D30, D40 and D50, flesh, fruit stems, bracts, leaves, petals, sepals and stamens were collected and stored at −80 °C.

2.2. Cloning of AcFT cDNA

Total RNA was extracted from apical meristems using Column Plant RNAout 2.0 kit (TIANDZ, Inc, China). First-strand cDNA was synthesized with M-MLV-Reverse transcriptase from TAKARA (Dalian, China) according to its instruction.

To clone the conserved region of *AcFT* cDNA, a pair of primers FT-partial-F (5′-GAC CGG AGA CCC GAG GTT GTA GAGC-3′) and FT-partial-R (5′-TGA GGT CGG AGG AAC TGA TCA TCT GAGA-3′) was designed according to the conserved regions of *FT* homologs from other plants using the Primer Premier 5 software. The PCR amplification was carried out under the following condition: 1 cycle at 94 °C for 3 min; 32 cycles at 94 °C for 0.5 min, 52 °C for 1 min, and 72 °C for 1 min; 1 cycle at 72 °C for 10 min. PCR products were isolated and cloned into pMD18-T Vector (TAKARA, Dalian, China) to sequence. The cloned sequence was used to design gene-specific primers to amplify the 5′ and 3′ ends of cDNA. The primers were 5′ FT-OUT (5′-GTA TTG AAG TTC TGA CGC CAG CCCG-3′) and 5′FT-IN (5′-TGC CCA AAA GAT GCT TCA GTT GTC GC-3′) for 5′ RACE; 3′FT-OUT (5′-CAT CGG ATC GTG TTC GTG CTA TTT CAA-3′) and 3′FT-IN (5′-GGC TGG CGT CAG AAC TTC AAT ACC CG-3′) for 3′ RACE. The first round PCR and the nested amplification were carried out according to the instruction of RACE cDNA Amplification Kit (Clontech, Dalian, China). The PCR products were cloned into pMD18-T vector and sequenced.

The full length cDNA of *AcFT* was obtained by PCR using FTQC-F (5′-ACT GGT TCT ACT TGA GTT CTT TTC ACC AG-3′) and FTQC-R (5′-ATT TAC AAT GTA TGG AGA AAA CAT AT-3′). Primers FTmq-F (5′-TGC TCTAGA CTA GCT AAG CTA GAG TGT GGT TGG TC-3′) and FTmq-R (5′-TCC CCCGGG GTG CTC TTT ATT AAT TGA CCT TGA TA-3′) contained the generated *Xba*I and *Sma*I recognition sites respectively to facilitate the transformation of *AcFT* into *Arabidopsis*. The cycle condition: 1 cycle at 94 °C for 3 min; 35 cycles at 94 °C for 0.5 min, 48 °C for 1.5 min and 72 °C for 1 min; a final extension at 72 °C for 10 min. The PCR products were cloned into pMD18-T vector and sequenced.

2.3. Sequence and phylogenetic analyses

Sequence chromatograms were examined and edited using Chromas Version 2.23. Related sequences were found using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). For determination of amino acid identities, sequences taken from the alignment were pairwise-compared using DNAMAN 6.0. Phylogenetic tree based on the protein sequences was constructed using DNAMAN 6.0. Gaps appearing in one sequence only were treated as non-constant characters. Molecular weight and isoelectric point of the gene were on-line analyzed with ExPASy (http://web.expasy.org/compute_pi/).

2.4. qRT-PCR analyses of the AcFT gene

To study transcription of the *AcFT* gene by qRT-PCR, total RNA of apical meristems, flesh, fruit stems, bracts, leaves, petals, sepals and stamens was extracted with Column Plant RNAout 2.0 kit. First-strand cDNA was synthesized with PrimeScript® RT Master Mix (TAKARA, Dalian, China) according to the user manual. These cDNAs were used as templates for qRT-PCR. Each template was applied to three replications for gene expression. Relative quantification was performed using the comparative Ct method when the target gene and the reference control gene had approximately equal amplification efficiency. The expression number in one tissue or stage was set to a value of 1 and subsequently expression levels were relative to this number.

qRT-PCR was carried out using SYBR® Premix Ex Taq™ kit (TAKARA, Dalian, China). Real time PCR reactions were performed in 25 μl mixtures. The mixture for one reaction contained 12.5 μl 1× SYBR Green PCR Master Mix with 0.5 μl ROXII as a reference dye for real time PCR, 1 μl 10 μM of forward primer, 1 μl 10 μM reverse primer and 100 ng of cDNA. No template controls were run to determine contamination and level of primer dimmer formation. To make it possible to compare gene expression levels in the different plant tissues, they were normalized to the expression of *Ac18SrRNA* in each tissue. Relative expression of *AcFT* in different plant tissues was obtained by dividing the average number of copies by the copy number of *Ac18SrRNA* for the same tissues.

qRT-PCR reactions were run on a Stratagene Mx3005P detection system (Stratagene 3005P, USA) using the following universal cycling conditions for all amplifications: 1 cycle of 30 s at 95 °C; 40 cycles of 5 s at 95 °C and 1 min at 55 °C. At the end, a dissociation stage was added: 30 s at 95 °C, 1 min at 55 °C and 30 s at 95 °C. Ct values were determined after automatic adjustment of the baseline and manual adjustment of the fluorescence threshold.

The primers used in this qRT-PCR were listed below: FTdl-up (5′-GCT CCA AGT CCC AGT TAC CCA A-3′), FTdl-dn (5′-GCT CAC AAT CTC CTG CCC AAA A-3′). *Ac18SrRNA* was used as the housekeeping gene. Ac18SrRNA-up (5′-ATG GTG GTG ACG GGT GAC-3′), Ac18SrRNA-dn (5′-AGA CAC TAA AGC GCC CGG TA-3′).

2.5. Arabidopsis transformation and transgenic plants analysis

AcFT was excised from the pMD18-T vector using *Xba*I and *Sma*I restriction enzymes and inserted into the vector pBI121 under the control of CaMV 35S promoter. After confirmation of the sequence, the plant expression vector was transformed into *Agrobacterium tumefaciens* strain GV3101 via the freeze-thaw method. Then the 35S::*AcFT* was transformed into *Arabidopsis thaliana* ecotype *Columbia* plants using floral dip method.

Transformants survived in the 1/2 times MS medium containing Km (50 mg/l) were further verified by PCR analysis. For PCR analysis, the Column Plant DNAout kit (TIANDZ, Inc, China) was used to isolate DNA from fresh leaves (100 mg) of T1 transgenic plants and non-transgenic plants.

3. Results

3.1. Cloning and sequence analyses of AcFT

A combined RT-PCR and RACE strategy was used to isolate *FT*-like gene from pineapple. The full-length gene designated *AcFT* (GenBank ID: HQ343233) was isolated. *AcFT* cDNA is 915 bp in length and contains an ORF of 534 bp, which encodes a protein of 177 aa. It also contains 79 bp 5′ and 294 bp 3′ untranslated regions and a poly(A) tail. Molecular weight and isoelectric point of the deduced protein were 19.9 kDa and 6.96 respectively. An alignment of the deduced protein sequence of *AcFT* and other *FT* homologs (Table 1) was performed using the DNAMAN 6.0 program (Fig. 1).

Phylogenetic analysis was conducted using the sequences in 'Table 1' and generated an unrooted tree by the observed divergency method with DNAMAN 6.0 program (Fig. 2). The phylogenetic tree showed that *AcFT* protein (*red-boxed*) was more closely related to

Table 1
List of sequences used in the alignment and phylogenetic analyses.

Taxa	FT-homologs	GenBank accession number
Carica papaya	CpFT	ACX85427
Malus × domestica	MdFT	ACL98164
Pyrus pyrifolia	PpFT2a	BAJ11577
Prunus mume	PmFT	BAH82787
Gossypium hirsutum	GhFT	ADK95113
Populus tremula	PtFT	ABD52003
Citrus unshiu	CiFT	BAF96644
Cymbidium goeringii	CgFT	ADI58462
Oncidium Gower Ramsey	OgFT	ACC59806
Oryza rufipogon	Hd3a	BAG72301
Helianthus annuus	HaFT	ADF32944
Chrysanthemum × morifolium	CmFT	ACX48949
Cucumis sativus	CsFT	BAH28253
Arabidopsis thaliana	AtFT	AAF03936
Solanum lycoper-sicum	SP3D	AAO31792
Triticum aestivum	TaFT	ACA25439
Lolium temulentum	LtFT	CBN73219
Festuca pratensis	FpFT	CBN73209
Hordeum vulgare subsp. Vulgare	HvFT1	ABV59396
Musa acuminata	MaFT3	ADP89470
Lolium perenne	LpFT3	ABC33722
Lolium perenne	LpFT	CBN73222
Phyllostachys meyeri	PmFT3	BAI49900
Hordeum vulgare subsp. Vulgare	HvFT3	ABD75336
Hordeum vulgare subsp. Vulgare	HvFT4	ABD75337
Hordeum vulgare subsp. Vulgare	HvFT5	ABM26903
Ananas comosus cv. *Comte de Paris*	*AcFT*	HQ343233

the other monocot *FT* proteins than to their dicot counterparts. *AcFT* protein was clustered into the same subgroup with *CgFT* (84%), *OgFT* (82%) and *Hd3a* (76%) which induced early flowering. Tamaki et al. (2007) reported that *Hd3a* protein is likely the florigen and induces flowering in *Arabidopsis* and rice.

3.2. qRT-PCR analyses of AcFT

To determine the detailed expression pattern of the *AcFT* in different tissues and stages, the mRNA levels were examined by qRT-PCR. The qRT-PCR analysis showed that the expression of *AcFT* was very low in apical meristems, bracts, petals and stamens, high in flesh and fruit stems, and moderate in sepals. There is no expression of *AcFT* in leaves (Fig. 3a).

The expression levels of *AcFT* in different stages (Fig. 3b) showed a little change on D10 and D20. It began to increase from D30 and reached the highest level on D40 when the multiple fruit and floral organs were forming. It remained high level on D50 because some fruitlets and floral organs were still forming.

3.3. Ectopic expression of AcFT in Arabidopsis

To investigate the function of *AcFT*, ectopic expression of *AcFT* in *Arabidopsis* plants was analyzed. Nine independent PCR-positive 35S::*AcFT* transgenic T1 plants were obtained (Fig. 4). All transgenic plants were phenotypically distinguishable from wild type plants. Firstly, these 35S::*AcFT* transgenic plants had only 8 to 10 small rosette leaves (Fig. 5a) while the wild type plants possessed over 16 big rosette leaves (Fig. 5b) when they flowered. The transgenic plants flowered 6.8 d earlier than wild type plants (Fig. 5c). Secondly, these 35S::*AcFT* plants had 3 to 6 inflorescences or branches (Fig. 5d), while the wild type plants had one inflorescence or branch (Fig. 5e). No obvious alteration of floral organs was observed in 35S::*AcFT* transgenic *Arabidopsis* plants.

4. Discussion

FT gene family is extraordinarily conservative in sequence, function and evolution across angiosperms (Aki et al., 2008; Hsu et al., 2006). The deduced *AcFT* protein displays all the characteristic features of the *FT* protein subfamily (Ahn et al., 2006) (Fig. 1). *FT* homologs play an important role in flower development. *FT* protein is the likely mobile long-distance signal which is transported from leaves, through the phloem, where it interacts with the bZIP transcription factor *FD*, and to the shoot meristem, and ultimately induces flowering in *A. thaliana* (Abe et al., 2005; Wigge et al., 2005). The expression of the *FT* gene and its homologs is the critical step of flowering in plants (Hiraoka et al., 2008). So far, many *FT* homologs

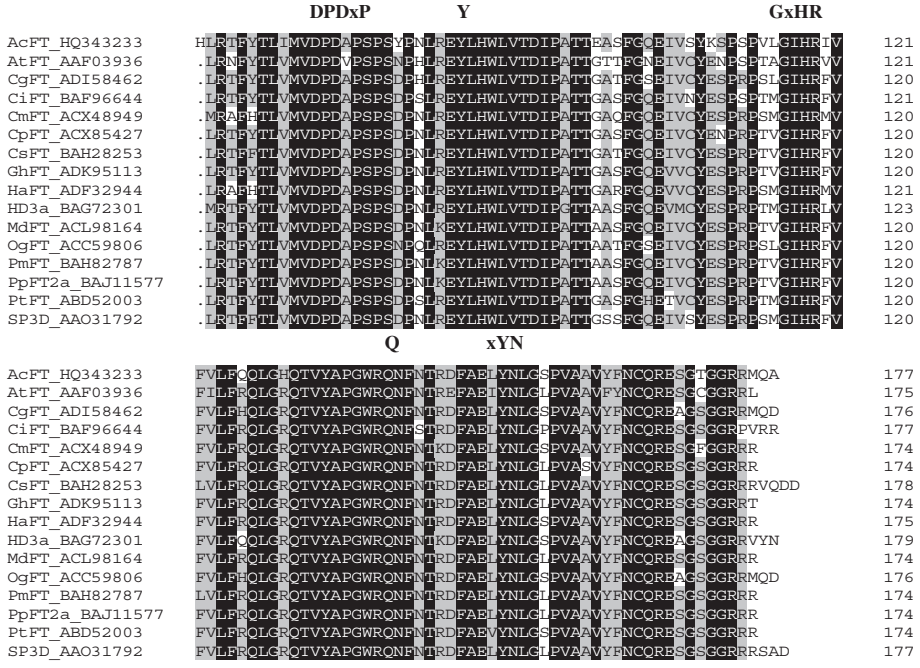

Fig. 1. Alignment of the deduced *AcFT* protein from *Ananas comosus* with other *FT*-like proteins. Identical amino acid residues are shaded in black, and similar residues in gray. *Dashes* denote gaps and *capital letters* above the alignment indicate functionally important conserved amino acids. The alignment was performed with the DNAMAN 6.0 program.

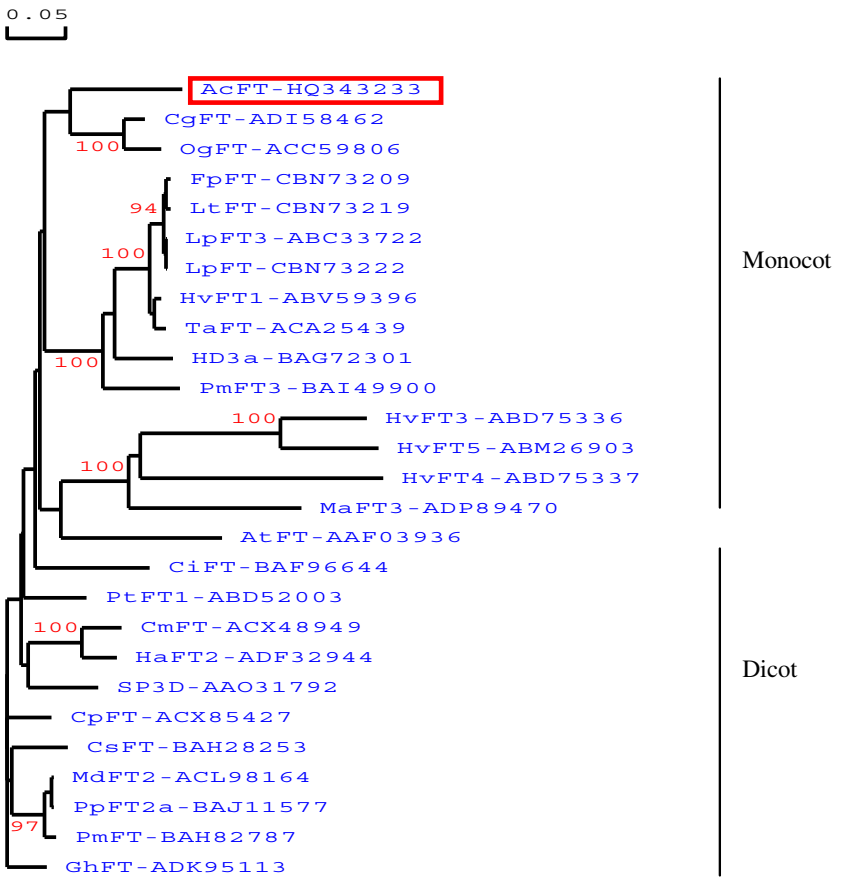

Fig. 2. Phylogenetic analysis of the *FT* homologs from different plant species. Protein sequences were obtained from the NCBI database. The numbers next to the nodes give bootstrap values from 1000 replicates and the branch lengths are proportional to the distance.

had been cloned from different plant species and ectopic overexpression of them, including rice *Hd3a*, tomato *SFT*, citrus *CiFT* and some others, in *Arabidopsis* significantly induced early flowering (Endo et al., 2005; Hemming et al., 2008; Kotoda et al., 2010; Lifschitz et al., 2006; Tamaki et al., 2007). The phylogenetic tree showed that *AcFT* protein, rice *Hd3a* were included in the same group. The result suggested that *AcFT* might potentially accelerate flowering.

The expression pattern of *FT*s is complicated. The expression levels of *FT*s are mostly high in leaves (Li et al., 2010; Wigge et al., 2005). In this work, *AcFT* had high expression in flesh, fruit stems and stamens, whereas there is no expression in leaves. Zong (2007) reported that *VvFT* had high expression during fruit development and no expression in leaves. The results above indicated that *FT* homologs play an important role not only in flower development but also in fruit development. Recent research has suggested that the *FT* protein, rather

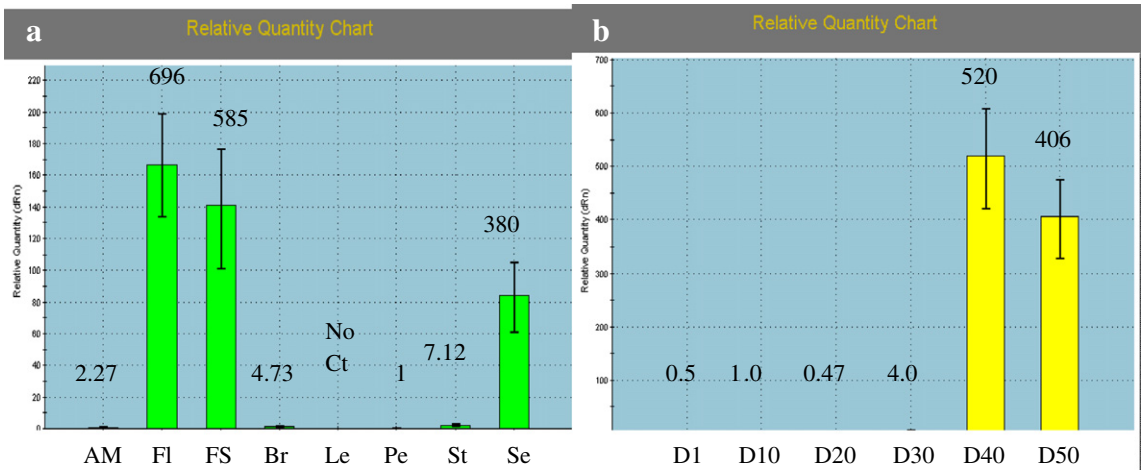

Fig. 3. Quantification of expression levels of the *AcFT* gene in different tissues (a) and stages (b) as determined by qRT-PCR analysis. The housekeeping gene *Ac18SrRNA* was used to normalize the amount of cDNAs added to the reaction. The relative quantification values of different tissues are showed above each vertical bar. *AM* apical meristems, *Fl* flesh, *FS* fruit stems, *Br* bracts, *Le* leaves, *Pe* petals, *St* stamens, *Se* sepals.

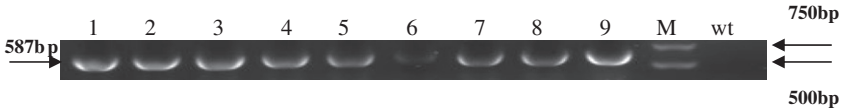

Fig. 4. PCR analysis. A 587 bp DNA fragment was amplified. 1 to 9, 35S::*AcFT* transgenic *Arabidopsis* plants. *M*, Marker DL2000. *wt*, wild type plant.

than *FT* transcript is the likely florigen (Corbesier et al., 2007). The ectopic expression of *AcFT* proved that *AcFT* can accelerate flowering and inflorescences or branches forming in *Arabidopsis*. Endo et al. (2005) reported that all of the 35S::*CiFT* transgenic citrus had a heavily branched tree shape.

Conflict of interest

The authors declare that they have no conflict of interest.

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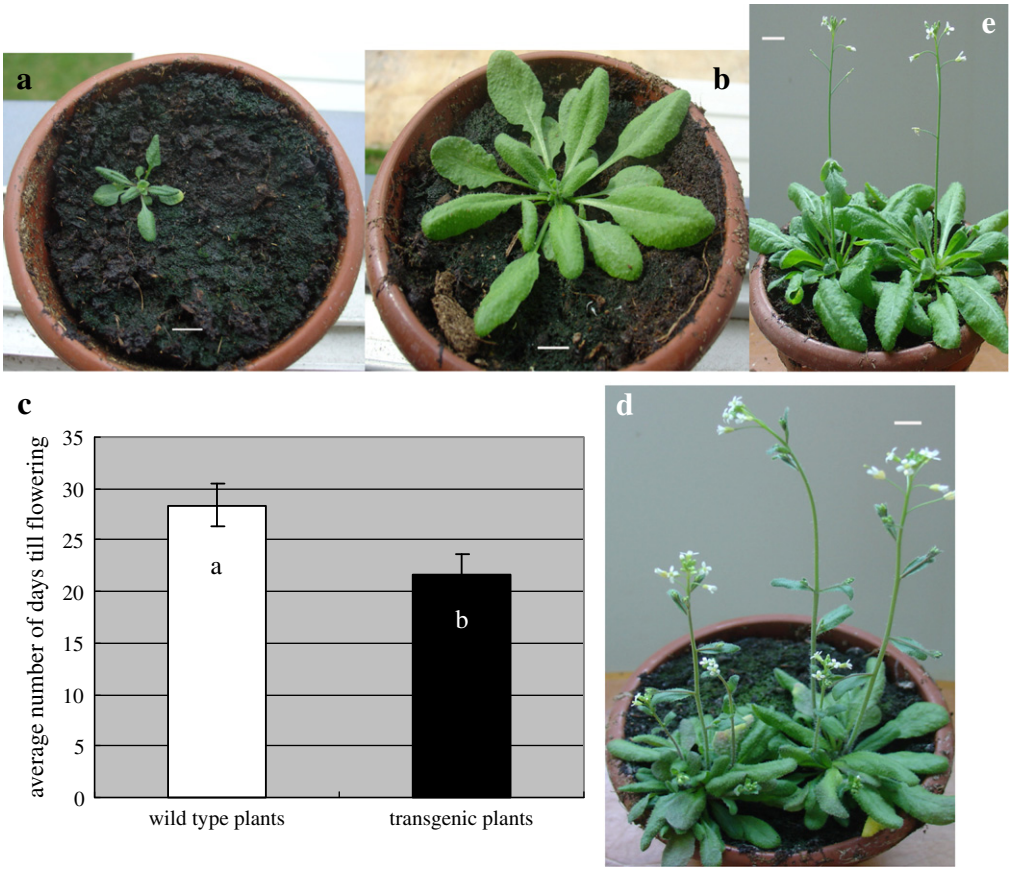

Fig. 5. The flower buds formation of the 35S::*AcFT* transgenic *Arabidopsis* plants (a) and the wild type plants (b). (c) Comparison of flowering times in transgenic *Arabidopsis* plants and wild type plants. The number of days to flowering was estimated from transplanting date to the appearance of the inflorescence. Data points represent averages with standard errors for each group. Letters indicate statistically significant differences between the groups (Student's *t*-test, $P<0.05$). (d) The 35S::*AcFT* transgenic *Arabidopsis* plant with 4 to 5 inflorescences. (e) The wild type plant with one inflorescence. *Scale bars* represent 1 cm.

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