BIOLOGIA PLANTARUM 54 (4): 639-646, 2010

Ectopic over-expression of two apple *Flowering Locus T* homologues, *MdFT1* and *MdFT2*, reduces juvenile phase in *Arabidopsis*

W.M. LI[1,2], Y. TAO[1], Y.X. YAO[1], Y.J. HAO[1] and C.X. YOU[1]*

State Key Laboratory of Crop Biology and College of Horticulture Science and Engineering,
Shandong Agricultural University, Tai-An, Shandong 271018, P.R. China[1]
South Subtropical Crop Research Institute, Chinese Academy of Tropical Agricultural Science,
Zhanjiang 524091, P.R. China[2]

Abstract

To get insight into mechanism by which apple tree (*Malus domestica* Borkh.) regulates flowering, two apple *flowering locus T* (*FT*) homologues, *MdFT1* and *MdFT2*, were isolated from the leaf cDNAs of cultivar Gala. The open reading frames (ORFs) of two *MdFTs* encoded 174 amino acids. The deduced amino acid sequence of *MdFT1* and *MdFT2* showed 94.3 % similarity to each other, while 72.6 and 76.0 % to AtFT protein, respectively. Semi-quantitative RT-PCR indicated their specific expression in leaves. Visualization of MdFT2-GFP fusion protein demonstrated its localization on membrane. Ectopic overexpression of either *MdFT1* or *MdFT2* in *Arabidopsis* significantly induced early flowering by activating the downstream flowering-related genes.

Additional key words: apple florigen, early flowering, RT-PCR.

Introduction

Many perennial woody plants have a long juvenile phase, spanning from several years to a few decades. In this period, any techniques can not induce to produce flowers or fruits (Hackett 1985, Martín-Trillo and Martínez-Zapater 2002, Poethig 1990). This characteristic may be an advantage for their fully growth and development so that enable them to be competent to adapt various environments. However, in fruit trees, it is a disadvantage severely hampering traditional breeding and genetic studies. In the case of apple (*Malus domestica* Borkh.), the breeding procedure consists of at least three steps: cross-pollination, seedling selection and regional trials before a variety that meets consumer demands is released. It usually takes 15 or more years. Fortunately, a progress has been achieved during the past decades (Blazquez and Weigel 2000, Colasanti and Sundaresan 2000, Simpson and Dean 2002, Yang *et al.* 2007).

Up to now, our understanding of the genetic control of flowering time mainly comes from studies in *Arabidopsis*. Several important *Arabidopsis* regulators that control flowering time have been isolated and characterized. These include *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), *APETALA1* (*AP1*), *FLOWERING LOCUS C* (*FLC*), *TERMINAL FLOWER 1* (*TFL1*) (Mandel *et al.* 1992, Weigel *et al.* 1992, Ohshima *et al.* 1997, Kobayashi *et al.* 1999, Michaels and Amasino 1999, Lee *et al.* 2000). In transgenic *Arabidopsis*, over-expressing the former four genes caused early flowering (Kobayashi *et al.* 1999, Lee *et al.* 2000, Mandel and Yanofsky 1995, Weigel and Nilsson 1995), whereas over-expression of the later two delayed flowering (Ohshima *et al.* 1997, Ratcliffe 1998). Mean- while, ectopic over-expression of *Arabidopsis* early- flowering genes in grass and woody plants caused early flowering in some cases, while did not in other cases (Nilsson and Weigel 1997, Rottmann *et al.* 2000, Penà *et al.* 2001, González-Schain and Suárez-López 2008). By contrast, ectopic over-expressing the homologues from woody plants usually induced early flowering in *Arabidopsis* (Kobayashi *et al.* 1999, Rottmann *et al.* 2000, Wada *et al.* 2002). Here, a possible explanation is that the

---

Received 18 April 2009, *accepted* 5 December 2009.
Abbreviations: Ct - cycle threshold; GFP - green fluorescent protein; LD - long-day; MS - Murashige and Skoog; ORF - open reading frame; PEPBs - phosphatidylethanolamine binding proteins; SD - short-day.
Acknowledgments: This work was supported by Program for New Century Excellent Talents in University (NCET-06-607) and Program for Changjiang Scholars and Innovative Research Team in University (IRT0635). The first two authors contributed equally to this paper.
* Corresponding author; fax: (+86) 538 8242364, e-mail: youchunxiang@sdau.edu.cn

inconsistent effects of these genes might be caused by some specific interactions between the exogenous genes and the host plants (Penà *et al.* 2001, Martín-Trillo and Martínez-Zapater 2002, Hsu *et al.* 2006). Hence, for genetic improvement it is necessary to isolate and characterize native genes.

Apple is one of the most economically important fruit tree crops in the world. However, little is known in apple tree about genes that control the transition from vegetative to reproductive phase and about the mechanism by which juvenility is modulated. Recently, FT protein, but not *FT* mRNA, has been proven to be a mobile signal migrated from leaves to the apical meristem to promote floral initiation in several plant species (Yang *et al.* 2007). *MdFT*, referring to *MdFT1* in this study, was isolated and first appeared in the paper by Kotoda and Wada (2005). More recently, its genomic organization and expression patterns were described by Hättasch *et al.* (2008). However, it is yet open question if *FT* homologous genes are involved in flowering regulation in apple tree. To answer this question, two *MdFT* genes, *MdFT1* and *MdFT2*, were isolated and identified in this study. Their role in promoting flowering was investigated by ectopic over-expression in *Arabidopsis*, and then the putative mechanism on its involvement in flowering control was discussed.

Materials and methods

Apple (*Malus domestica* Borkh.) leaves, flowers and young fruits of fruit-bearing trees were collected from cultivar Gala in the experimental orchard of Shandong Agricultural University, China. Callus tissues were generated from leaf explants in dark.

Arabidopsis thaliana ecotype Columbia (Col) was used in experiments. Seeds were surface sterilized with bleach and sown on Murashige and Skoog (1962; MS) agar medium containing 3 % sucrose. After being stored in dark at 4 °C for 3 d, the seeds were germinated. Seedlings (15-d-old) were then transferred to soil and grown in a growth chamber at ~22 °C under long day (LD; 16-h photoperiod) or short day (SD; 8-h photoperiod). Flowering time was measured by scoring the number of rosette leaves of plants grown under SD conditions.

Total RNA was extracted from apple tissues and *Arabidopsis* seedlings using *Trizol* reagent (*Invitrogen Life Technologies*, San Diego, USA) according to the manufacturer's instructions. RNA from each sample (2 µg) was reverse-transcribed by oligo(dT) primer with reverse transcriptase. Gene-specific sense primer MdFT5 (5'-ATGCCTAGGGATAGGGACC-3') and antisense primer MdFT3 (5'-TTATCTTCTCCTTCCACCGG-3'), and sense primer AtFT5 (5'-GATGTCTATAA ATATAAGAGACCCTC-3') and antisense primer AtFT3 (5'-TCTAAAGTCTTCTTCCTCCGCAGC-3') were used to amplify *MdFT* and *AtFT* open-reading frames, respectively. The amplified fragments were cloned into pMD18 cloning vector (*TaKaRa Biomedicals*, Dalian, China) and sequenced for confirmation.

Public databases were searched using various *Arabidopsis* and *Oryza sativa FT*-related flowering-time genes as queries. Gene names and their GenBank accessions used were as follows: *AmCEN* (S81193.1) from *Antirrhinum majus*; *AtATC* (NM_128315.2), *AtBFT* (NM_125597), *AtFT* (NM_105222), *AtMFT* (NM_101672), *AtTFL1* (NM_120465) and *AtTSF* (NM_118156.1) from *Arabidopsis thaliana*; *CmFTL1* (DQ865290) and *CmFTL2* (DQ865291) from *Cucurbita maxima*; *BnTFL1-1* (AB017525) from *Brassica napus*; *CmoFTL1* (EF462211) and *CmoFTL2* (EF462212) from *Cucurbita moschata*; *CiFT* (AB027456) from *Citrus sinensis*; *LeSP* (U84140), *LeSFT* (AY186735.1) and *LeSP5G* (AY186736) from *Lycopersicon esculentum*; *MdFT1*, *MdFT2*, *MdTFL1-1* (BAD06418) and *MdTFL1-2* (BAD10967) from *Malus domestica*; *NtCET1* (AF145259), *NtCET2* (AF145260) and *NtCET4* (AF145261) from *Nicotiana tabacum*; *OsFT* (AC136448), *OsHd3a* (NM_185584), *OsMFT1* (NM_183536) and *OsRCN2* (XM_472397) from *Oryza sativa*; *PtFT1* (poplar database number LGVII000284) from *Populus trichocarpa*; *VvTFL1* (AF378127) from *Vitis vinifera*; and *TaFT* (AY705794) from *Triticum aestivum*. Multiple alignments of the predicted amino acid sequences were created using software *DNASTAR* and the resulting alignment was used to generate the phylogenetic tree by the Neighbor-joining method with the bootstrap analysis of 1000 replicates.

Semi-quantitative RT-PCR was conducted to analyze *MdFTs* expression in different apple organs with primers MdFT5 and MdFT3 using the cDNAs of different samples as templates. Ribosomal *Md18S* RNA was used as loading control. PCR conditions were as follows: 3 min at 94 °C (one cycle), 30 s at 94 °C; 30 s at 53.5 °C and 45 s at 72 °C (30 cycles). The PCR products were run on 1.0 % (m/v) agarose gel and detected using an image analyzer (white/UV transilluminator).

To produce *35S::MdFT1, 35S::MdFT2* and *35S::AtFT* sense constructs, the cloning vectors were cut with *Xba*1 and *Sac*I to release the *MdFT1, MdFT2* or *AtFT* full-length cDNA fragments. After gel purification, *MdFT1, MdFT2* or *AtFT* genes were ligated in sense orientation into the *pBI121* expression vector (*TaKaRa Biomedicals*), digested with the same restriction enzymes, downstream of *CaMV 35S* promoter. The expression constructs were transferred into the *Agrobacterium tumefaciens* strain LBA3101 with a freeze-thaw method as described by Chen *et al.* (1994). Transgenic *Arabidopsis* plants were generated following a floral-dip method according to Clough and Bent (1998).

The *MdFT2* ORF without stop codon was obtained by RT-PCR using the following primers containing the digestion site (underlined): forward primer 5'-GCT <u>GGATCC</u>ATGCCTAGGGATAGGGACC-3'; reverse primer 5'-GCTGGATCCTCTTCTCCTTCCACCGG-3'. The resulting PCR fragment was digested with *Bam*HI and cloned into the binary vector pBIN down-stream of the *CaMV 35S* promoter. The resultant *MdFT2-GFP* construct was introduced into *Agrobacterium* strain LBA4404 and transformed into onion epidermal cells which were pre-incubated on MS agar plates in light at 22 °C for 24 h. Infected tissues were incubated on the same conditions for another 24 h, followed by monitoring the subcellular localization of GFP with a confocal laser-scanning microscope (*Zeiss LSM 510 META*, Jena, Germany).

Quantitative real-time PCR was performed in triplicates with *SYBR Green* PCR master mix (*Applied Biosystems*, Foster City, USA) using an *iQ5* multicolor real-time PCR detection system (*BioRad*, Hercules, USA). Efficiency of each pair of primers was determined based on its standard curve obtained from a series of 4-fold diluted template cDNAs. The difference between the cycle threshold (Ct) of target genes and the Ct of control primers ($\Delta Ct = Ct_{target\ gene} - Ct_{control}$) was used to obtain the normalized expression of target genes. Primer sequences used for different flowering time genes were as follows: MdFTs (5'-ACGGATATTCCAGCAACAACTGC-3') and MdFTan (5'-GCGAAACAGCACAAAAACAA-3') for *MdFT1* and *MdFT2*; AtFTs (5'-AACAATCAACACAGAGAAACCACCT-3') and AtFTan (5'-GATCAAGAACGTCTCCAACAACTCT-3') for *AtFT*; AtSOC1s (5'-CTCCTCCTATATCTCTACCTATAC-3') and AtSOC1an (5'-GTCACTTGTCTGCTTGTTG-3') for *AtSOC1*; AtLFYs (5'-GTGGAACCCAACGAGAGCA-3') and AtLFYan (5'-ACCAAGTCGCATCCCAAAAG-3') for *AtLFY*; AtAP1s (5'-AGCAGAACCAAGGCCACAA-3') and AtAP1an (5'-TGAGAAAAGGAGATGGCTGATG-3') for *AtAP1*; AtAGs (5'-TCGCACTCATCGTCTTCTC-3') and AtAGan (5'-GGCTGATTCTTGTTGATAATACTG-3') for *AtAG*; At18Ss (5'-GGAGTTTCAACACATTCTTCGTG-3') and At18San (5'-CCAATCGCCTACCAATACCTTT-3') for *At18S.*

Results

Blast found that a *FT*-like full-length cDNA sequence was already registered in GenBank with accession number AB161112 and named as *MdFT*, however, its function is not characterized yet. To get insight into the role of apple *FT*-like genes in flowering regulation, RT-PCR was conducted with AB161112 specific primers to amplify the full-length cDNA of *MdFT* from leaf cDNAs of apple cv. Gala. As a result, a single band was obtained. Following inserting into the cloning vector, six clones were randomly selected for sequencing. Interestingly, those clones fell into two groups depending on their sequences. The sequences were highly similar or exactly same to each other in one group, but relatively different between two groups. Therefore, two different genes existed in the PCR product, and then were designated as *MdFT1* and *MdFT2*, respectively. Their ORFs have the same size and encoded 174 amino acids. Alignment analysis of amino acid sequence showed that MdFT1 was completely identical to AB161112-encoded protein, while MdFT2 shared 94.3 % identity to MdFT1. Both MdFT1 and MdFT2 exhibited high similarity to FT proteins of other plant species (Fig. 1), with 72.6 and 76.0 % identities to AtFT, respectively.

As well known, plant FTs belong to CETS protein family which contains conserved amino acid residues or motifs that identical to mammalian phosphatidylethanolamine binding proteins (PEBPs). In MdFT1 and MdFT2, a D-P-D-x-P motif ranges from residue 70 to 74, followed by a histidine residue positioned at 86, and a G-x-H-R motif runs from residue 116 to 119. These motifs are exactly conserved in other members of the CETS family (Fig. 1).

Phylogenetic analysis among the deduced amino acid sequences of FT/TFL1-like proteins from different plants showed that MdFT1 and MdFT2 were grouped into the same cluster with other FT-like proteins which shorten juvenile phase and induce early flowering (Fig. 2). This result suggests that *MdFT1* and *MdFT2* may potentially accelerate flowering.

Due to the high similarity between *MdFT1* and *MdFT2*, it is hard to design specific primers distinguishing one gene from another. Therefore, the total transcripts of *MdFT1* and *MdFT2* were detected with the primers shared by two genes by semi-quantitative RT-PCR. The result indicated that *MdFT* transcripts were expressed specifically in leaf, but not in other samples tested such as flower, young fruit and callus (Fig. 3*A*).

To observe the *in vivo* localization of MdFT protein, MdFT2, which was more powerful in inducing early flowering in transgenic *Arabidopsis* than MdFT1, was fused in-frame to the N-terminal side of the green fluorescent protein (GFP) and transiently expressed under control of the *CaMV 35S* promoter in onion epidermis cells. It was found that GFP fluorescence was presented predominately on membranes, while GFP control constitutively full of the whole cell (Fig. 3*B*).

To determine the function of *MdFTs* in flowering regulation, full-length cDNAs of *MdFT1* and *MdFT2* were inserted in the sense-oriented direction into the plant expression vector *pBI121*, fusing downstream to the *CaMV 35S* promoter, then independently introduced into wild-type *Arabidopsis* plants by *Agrobacterium*-mediated transformation. In parallel, *AtFT* was transformed by the same way as a positive control. Under LD conditions, most kanamycin-resistant transgenic seedlings exhibited extremely early flowering even directly flowering in the selection medium and thus failed to produce T1 seeds (data not shown). To harvest seeds, transgenic plants were selected under SD conditions. As a result, more than 10 independent kanamycin-resistant T1 transgenic plants for each *35S::AtFT*, *35S::MdFT1* and *35S::MdFT2*, respectively, were selected for further investigation.

Under SD conditions, wild-type *Arabidopsis* plants flowered with 61 - 73 (average 62.38 ± 3.85 for 8 WT plants) rosette leaves. However, every transgenic line for each gene flowered earlier than the wild type. The earliest flowering appeared in *35S::AtFT* transgenic plants bearing only 2 - 5 (3.50 ± 1.09 for 8 transgenic plants) rosette leaves, while the latest in *35::MdFT1* plants with 7 - 12 (8.93 ± 1.54 for 14 transgenic plants) rosette leaves. *35S::MdFT2* transgenic plants flowered early to a medium extent with 3 - 9 (5.41 ± 1.41 for 32 transgenic plants) rosette leaves. The morphology observation also showed that *35S::MdFT2* transgenic plants generally flowered earlier than *35::MdFT1* plants, suggesting that the *MdFT2* was more powerful in promoting *Arabidopsis* flowering than *MdFT1*. Therefore, the ectopic overexpression of both *MdFT1* and *MdFT2* accelerated flowering in *Arabidopsis*, suggesting their potential function associated with juvenility in apple tree.

To get insight into how *MdFTs* initiate flowering in *Arabidopsis*, homozygous T2 transgenic lines were selected from the T1 self-cross population for each gene, and then sown for quantitative characterization of flowering time. The transcript levels of four *FT*-downstream flowering-related genes, *i.e. AtSOC1*, *AtLFY*, *AtAP1* and *AtAG*, were examined in response to the ectopic over-expression of *AtFT*, *MdFT1* and *MdFT2*. The earliest flowering T2 lines for *35S::AtFT* and *35::MdFT1*, respectively, as well as three *35S::MdFT2* T2

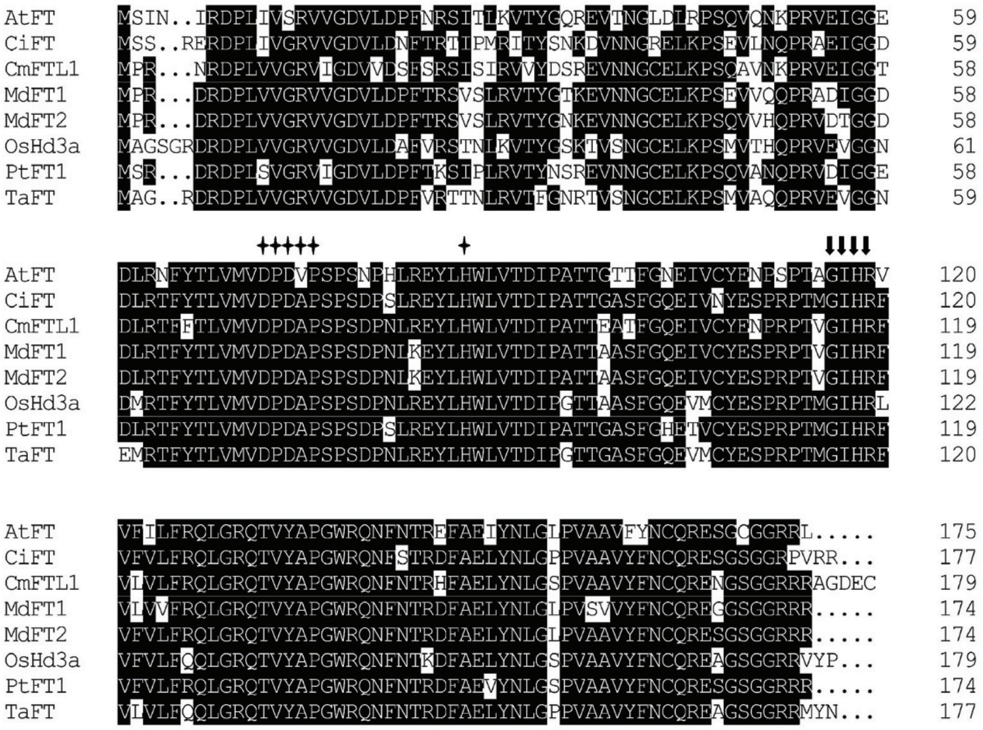

Fig. 1. Comparison of amino acid sequences encoded by *MdFT1*, *MdFT2* and other six homologue *FT* genes from different species. *Black boxes* indicate identity; *crosses* and *arrowheads* stand for D-P-D-x-P and G-x-H-R motif of mammalian PEBPs, respectively.

Table 1. Quantitative real-time RT-PCR of transgenes and endogenous genes in WT and transgenic *Arabidopsis*. The relative expression of *AtFT, AtLFY, AtAP1, AtAG* and *AtSOC1* were relative to those of WT, respectively, and *MdFTs* to *18S* rRNA in 30-d-old seedlings. Plants of each WT, *35S::AtFT* and *35S::MdFT1* as well as three *35S::MdFT2* lines with the earliest (3 - 5 rosette leaves), earlier (6 - 7 rosette leaves) and early (8 - 9 rosette leaves) flowering phenotypes, respectively, were grown under SD conditions.

Genes	WT	*35S::AtFT*	*35S::MdFT1*	*35S::MdFT2*		
				earliest	earlier	early
AtFT	1.0 ± 0.3	62.1 ± 3.8	-	-	-	-
AtLFY	1.0 ± 0.1	148.7 ± 24.7	1.4 ± 0.1	17.3 ± 4.1	3.0 ± 0.3	2.6 ± 0.2
AtAP1	1.0 ± 0.3	83.3 ± 4.2	10.8 ± 1.0	17.7 ± 2.3	12.3 ± 1.8	11.8 ± 1.9
AtAG	1.0 ± 0.3	31.4 ± 3.0	23.1 ± 2.1	4.5 ± 0.3	17.0 ± 1.5	14.6 ± 1.6
AtSOC1	1.0 ± 0.4	4.2 ± 0.4	3.3 ± 0.6	1.9 ± 0.3	3.8 ± 1.0	4.7 ± 0.7
MdFTs	-	-	21.3 ± 9.4	16.9 ± 2.8	8.7 ± 1.8	3.3 ± 0.8

lines with the earliest (3 - 5 rosette leaves), earlier (6 - 7 rosette leaves) and early (8 - 9 rosette leaves) flowering phenotypes, respectively, were chosen for examination with real time RT-PCR. All leaf samples were harvested from 30-d-old seedlings grown under SD conditions before flower buds appeared. The results demonstrated that the transcript abundance of each gene was much higher in transgenic lines than in the wild type (Table 1). Therefore, overexpression of *MdFTs* accelerated flowering by modulating flowering-related genes in *Arabidopsis*, suggesting that FT proteins function in a conserved regulation cascade to promoter flowering in different plant species. In addition, it was found among three *35S::MdFT2* transgenic lines that the expression levels of *MdFT2* were positively related with the flowering time, *i.e.* the higher expression, the earlier flowering, further suggesting the function of *MdFT2* in promoting flowering.

Discussion

Mammalian phosphatidylethanolamine binding proteins (PEPBs) bind phospholipids *in vitro* and inhibit the activity of Raf-1 kinase *in vivo* (Schoentgen and Jollès 1995, Yeung *et al.* 1999). These proteins share several regions of notable sequence homology including a D-P-D-x-P motif followed at some distance by a histidine residue and then a G-x-H-R motif, all of which contribute to the conformation of the ligand-binding site (Banfield and Brady 2000). There is also a group of proteins sharing homology to PEBPs in the plant kingdom, which compose CETS family with a suggested function of mediators in a variety of signaling pathways including flowering time control mediated by FT proteins (Pnueli *et al.* 2001). In this study, it was found that both MdFT1 and MdFT2 contained a D-P-D-x-P motif followed by a histidine residue and a G-x-H-R motif, that are also conserved in other members of the CETS family.

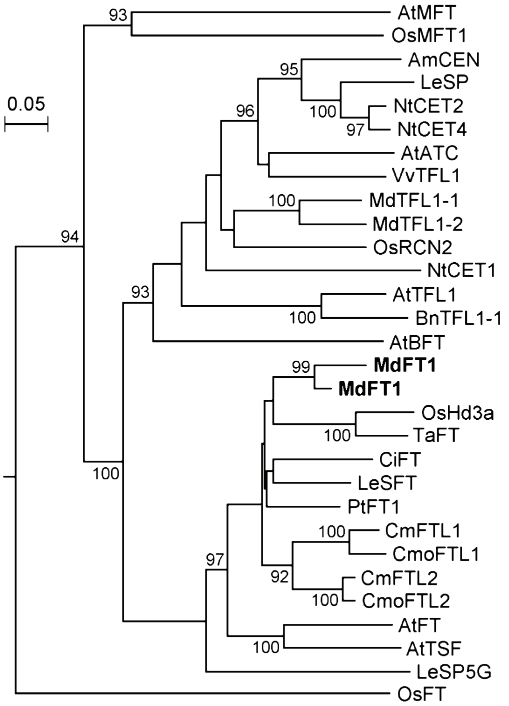

Fig. 2. Phylogenetic relationship of FT/TFL1-like protein. The numbers next to the nodes give bootstrap values from 1000 replicates. The genes and their accession numbers are found in Materials and methods.

Two *MdFT* genes are highly similar to each other, but 5.7 % difference between them suggests that they should be on different loci in apple genome, maybe arising from its genetic background of high heterozygosity and allopolyploidy (Korban and Chen 1992). A recent study also showed that *Populus* has two *FT-like* genes in each species (Hsu *et al.* 2006). Generally, the presence of multi-alleles is considered as a highly adaptive trait in perennial woody plants because it ensures the reproduction successful in every annual cycle. Our transgenic assay also proved that *MdFT1* and *MdFT2* offered a non-identical service on the capacity to promote flowering, an observation coincided with other multi-alleles gene in different species (Wada *et al.* 2002, Lin *et al.* 2007).

The striking advances in genetic control of flowering in *Arabidopsis* have shown a distinct pathway: *CONSTANS* (*AtCO*), a critical gene that promotes flowering in response to long days (Fowler *et al.* 1999, Kobayashi *et al.* 1999, Park *et al.* 1999), activates *AtSOC1* directly and through *AtFT* promotes flowering (Onouchi et al. 2000, Yoo *et al.* 2005), whereas *AtLFY* that directly triggers *AtAP1*, *AtAG* and *AtAP3* expression acts at least in part downstream of *AtSOC1* (Ng and Yanoliky 2000, Yu *et al.* 2002). According to these clues, we chose *AtLFY*, *AtAP1*, *AtSOC1* and *AtAG* as markers to examine whether *MdFT1* and *MdFT2* positively regulate their expressions. Consistent with the previous studies, our findings showed that ectopic overexpression of either *MdFT1* or *MdFT2* promoted flowering in *Arabidopsis* by up-regulating those downstream genes.

Since several reports verified that FT protein is mobile as a long-range flowering stimulus (Lifschitz *et al.* 2006, Corbesier *et al.* 2007, Jaeger and Wigge 2007, Lin *et al.* 2007, Mathieu *et al.* 2007, Tamaki *et al.* 2007), little doubts have been remained about that FT acts as a florigen. Abe *et al.* (2005) found that *Arabidopsis* FT moved from leaves to shoot apex where it interacted with FD and formed FT-FD complex, which in turn triggers *AP1* transcription in nucleus, and that FT-EGFP was localized in the nucleus and cytoplasm. In tomato, however, FT ortholog SFT (SINGLE-FLOWER TRUSS) was localized primarily in the nucleus, although weak cytoplasmic staining was detected as well. Meanwhile, unlike *Arabidopsis* FD, the tomato FD homolog SPGB expressed in leaves, making the event of SFT interacting with SPGB happened in leaves. They therefore suggest that SFT triggers downstream systemic signals that regulate growth and flowering (Lifschitz *et al*. 2006). Therefore, FT orthologs from different plant species are different in subcellular localization and even in the way by which they regulate plant growth and flowering.

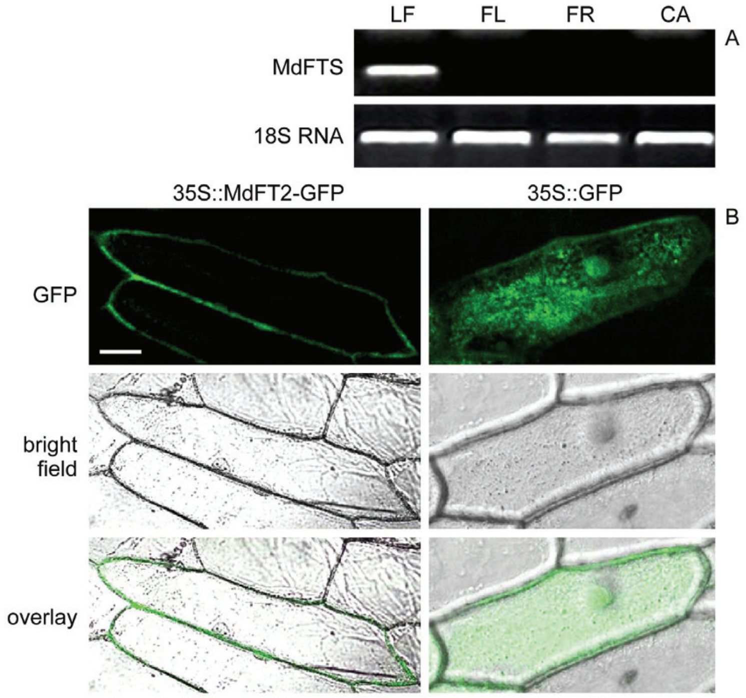

Fig. 3. Gene expression and protein subcellular localization. *A* - Semi-quantitative RT-PCR amplification of *MdFTs* with the cDNA as templates from leaf (LF), flower (FL), fruit (FR) and callus (CA) tissue of apple Gala. Ribosomal *Md18S* RNA was loading as control. *B* - Subcellular localization of MdFT2-GFP fusion protein. Transient GFP expression in onion epidermal cells transformed with constructs harboring *35S::MdFT2-GFP* or *35S::GFP* (as a control). GFP fluorescence was detected constitutively in the whole cell for *35S::GFP* construct, but specifically on membrane for *35S::MdFT2-GFP* construct (bar = 50 µm).

Up to now, the schematic flow of flowering genes in woody plants, especially in perennial woody plants which show great differences in growth habit, shape-size, reproduction behavior with *Arabidopsis*, is unknown yet. As a perennial woody plant, apple has a long juvenile phase. Although an investigation, which aims at identifying the relationships between the expression of flowering genes and the flower induction and initiation in apple, proves that it has a certain similarity to *Arabidopsis* (Hättasch *et al*. 2008), the detailed molecular mechanism by which FT proteins regulate apple flowering remains far from elucidation. In addition, considering the genetic background of high heterozygosity and allopolyploidy (Korban and Chen 1992), it could be hypothesized that apple genome may encode multiple forms of FT proteins to adapt the perennial growth habits and reproduction behavior. In this point, it is an interesting question whether there is apple FT protein with subcellular localization different from its *Arabidopsis* and tomato counterparts, and how it functions. In this study, our result indicated that MdFT2 protein was predominantly distributed on cytoplastic membrane, distinguishing from the previous findings in herbaceous plants. Further investigation is still needed to elucidate why it is localized in this way and how it regulates the expression of downstream genes.

References

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., Araki, T.: FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. - Science **309**: 1052-1056, 2005.

Banfield, M.J., Brady, R.L.: The structure of *Antirrhinum centroradialis* (CEN) protein suggests a role as a kinase regulator. - J. mol. Biol. **297**: 1159-1170, 2000.

Blazquez, M.A., Weigel, D.: Integration of floral inductive signals in *Arabidopsis*. - Nature **404**: 889-892, 2000.

Chen, H., Nelson, R.S., Sherwood, J.L.: Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. - Biotechniques **16**: 664-670, 1994.

Clough, S.J., Bent, A.F.: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis* *thaliana*. - Plant J. **16**: 735-743, 1998.

Colasanti, J., Sundaresan, V.: 'Florigen' enters the molecular age: long-distance signals that cause plants to flower. - Trends Biochem. Sci. **25**: 236-240, 2000.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., Coupland, G.: FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. - Science **316**: 1030-1033, 2007.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Coupland, G., Putterill, J.: *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. - EMBO J. **18**: 4679-4688, 1999.

González-Schain, N.D., Suárez-López, P.: *CONSTANS* delays flowering and affects tuber yield in potato. - Biol. Plant. **52**: 251-258, 2008.

Hackett, W.P.: Juvenility, maturation, and rejuvenility in woody plants. - Hort. Rev. **7**: 109-155, 1985.

Hättasch, C., Flachowsky, H., Kapturska, D., Hanke, M.V.: Isolation of flowering genes and seasonal changes in their transcript levels related to flower induction and initiation in apple (*Malus domestica*). - Tree Physiol. **28**: 1459-1466, 2008.

Hsu, C.Y., Liu, Y.X., Luthe, D.S., Yuceer, C.: Poplar *FT2* shortens the juvenile phase and promotes seasonal flowering. - Plant Cell **18**: 1846-1861, 2006.

Jaeger, K.E., Wigge, P.A.: *FT* protein acts as a long-range signal in *Arabidopsis*. - Curr. Biol. **17**: 1050-1054, 2007.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., Araki, T.: A pair of related genes with antagonistic roles in mediating flowering signals. - Science **286**: 1960-1962, 1999.

Korban, S.S., Chen, H.: Apple. - In: Hammerschlag, F.A., Litz, R.E. (ed.): Biotechnology of Perennial Fruit Crops, Biotechnology in Agriculture. Vol. 8. Pp. 203-227. Cambridge University Press, Cambridge 1992.

Kotoda, N., Wada, M.: *MdTFL1*, a *TFL1*-like gene of apple, retards the transition from the vegetative to reproductive phase in transgenic *Arabidopsis*. - Plant Sci. **168**: 95-104, 2005.

Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M., Lee, I.: The *AGAMOUS-LIKE 20* MADS domain protein integrates floral inductive pathways in *Arabidopsis*. - Genes Dev. **14**: 2366-2376, 2000.

Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., Alvarez, J.P., Eshed, Y.: The tomato *FT* ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. - Proc. nat. Acad. Sci. USA **103**: 6398-6403, 2006.

Lin, M.K., Belanger, H., Lee, Y.J., Varkonyi-Gasic, E., Taoka, K., Miura, E., Xoconostle-Cázares, B., Gendler, K., Jorgensen, R.A., Tony, B.P., Lough, J., Lucas, W.J.: FLOWERING LOCUS T protein may act as the long distance florigenic signal in the cucurbits. - Plant Cell **19**: 1488-1506, 2007.

Mandel, M.A., Gustafson-Brown, C., Savidge, B., Yanofsky, M.F.: Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. - Nature **360**: 273-277, 1992.

Mandel, M.A., Yanofsky, M.F.: A gene triggering flower formation in *Arabidopsis*. - Nature **377**: 522-524, 1995.

Martín-Trillo, M., Martínez-Zapater, J.M.: Growing up fast: manipulating the generation time of trees. - Curr. Opin. Biotechnol. **13**: 151-155, 2002.

Michaels, S.D., Amasino, R.M.: *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. - Plant Cell **11**: 949-956. 1999.

Mathieu, J., Warthmann, N., Kuttner, F., Schmid, M.: Export of *FT* protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. - Curr. Biol. **17**: 1055-1060, 2007.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Ng, M., Yanoliky, M.F.: Three ways to learn the ABCs. - Curr. Opin. Plant Biol. **3**: 47-52, 2000.

Nilsson, O., Weigel, D.: Modulating the time of flowering. - Curr. Opin. Biotechnol. **8**: 195-199, 1997.

Ohshima, S., Murata, M., Sakamoto, W., Ogura, Y., Motoyoshi, F.: Cloning and molecular analysis of the *Arabidopsis* gene *Terminal Flower 1*. - Mol. gen. Genet. **254**: 186-194, 1997.

Onouchi, H., Igeno, M.I., Perilleux, C., Graves, K., Coupland, G.: Mutagenesis of plants overexpressing *CONSTANS* demonstrates novel interactions among *Arabidopsis* flowering-time genes. - Plant Cell **12**: 885-900, 2000.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S., Kim, H.J., Kay, S.A., Nam, H.G.: Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis GIGANTEA* gene. - Science **285**: 1579-1582, 1999.

Penà, L., Martín-Trillo, M., Juárez, J., Pina, J.A., Navarro, L., Martínez-Zapater, J.M.: Constitutive expression of *Arabidopsis LEAFY* or *APETALA1* genes in citrus reduces their generation time. - Nat. Biotechnol. **19**: 263-267, 2001.

Pnueli, L., Gutfinger, T., Hareven, D., Ben-Naim, O., Ron, N., Adir, N., Lifschitz, E.: Tomato SP-interacting proteins define a conserved signaling system that regulates shoot architecture and flowering. - Plant Cell **13**: 2687-2702, 2001.

Poethig, R.S.: Phase change and the regulation of shoot morphogenesis in plants. - Science **250**: 923-930, 1990.

Ratcliffe, O.J., Amaya, I., Vincent, C.A., Rothstein, S., Carpenter, R., Coen, E.S., Bradley, D.J.: A common mechanism controls the life cycle and architecture of plants. - Development **125**: 1609-1615, 1998.

Rottman, W.H., Meilan, R., Sheppard, L.A., Brunner, A.M., Skinner, J.S., Ma, C., Cheng, S., Jouanin, L., Pilate, G., Strauss, S.H.: Diverse effects of overexpression of *LEAFY* and *PTLF*, a poplar (*Populus*) homolog of *LEAFY*/*FLORICAULA*, in transgenic poplar and *Arabidopsis*. - Plant J. **22**: 235-245, 2000.

Schoentgen, F., Jollès, P.: From structure to function: possible biological roles of a new widespread protein family binding hydrophobic ligands and displaying a nucleotide binding site. - FEBS Lett. **369**: 22-26, 1995.

Simpson, G.G., Dean, C.: *Arabidopsis*, the rosetta stone of flowering time? - Science **296**: 285-289, 2002.

Tamaki, S., Matsuo, S., Wong, H.L., Yokoi, S., Shimamoto, K.: *Hd3a* protein is a mobile flowering signal in rice. - Science **316**: 1033-1036, 2007.

Wada, M., Cao, Q.F., Kotoda, N., Soejima, J.I., Masuda, T.: Apple has two orthologues of *FLORICAULA*/*LEAFY* involved in flowering. - Plant mol. Biol. **49**: 567-577, 2002.

Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F., Meyerowitz, E.M.: *LEAFY* controls floral meristem identity in *Arabidopsis*. - Cell **69**: 843-859, 1992.

Weigel, D., Nilsson, O.: A developmental switch sufficient for flower initiation in diverse plants. - Nature **377**: 495-500, 1995.

Yang, Y.J., Klejnot, J., Yu, X.H., Liu, X.M., Lin, C.T.: Florigen (II): It is a mobile protein. - J. Integr. Plant Biol. **49**:

1665-1669, 2007.

Yeung, K., Seitz, T., Shengfeng, L., Janosch, P., McFerran, B., Kaiser, C., Fee, F., Katsanakis, K.D., Rose, D.W., Mischak, H., Sedivy, J.M., Kolch, W.: Suppression of Raf-1 kinase activity and MAP kinase signaling by RKIP. - Nature **401**: 173-177, 1999.

Yoo, S.K., Chung, K.S., Kim, J., Lee, J.H., Hong, S.M., Yoo, S.J., Yoo, S.Y., Lee, J.S., Ahn, J.H.: *CONSTANS* activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* through *FLOWERING LOCUS T* to promote flowering in *Arabidopsis*. - Plant Physiol. **139**: 770-778, 2005.

Yu, H., Xu, Y.F., Tan, E.L., Kumar, P.P.: *AGAMOUS-LIKE 24*, a dosage-dependent mediator of the flowering signals. - Proc. nat. Acad. Sci. USA **99**: 16336-16341, 2002.