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# *MdTFL1*, a *TFL1*-like gene of apple, retards the transition from the vegetative to reproductive phase in transgenic *Arabidopsis*

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**Abstract**

Unlike herbaceous plants, fruit trees such as the apple (*Malus* × *domestica* Borkh.) flower and set fruit only after an extended juvenile phase lasting several years. While studying juvenility in apple trees, we cloned *Malus domestica TFL1* (*MdTFL1*), a gene homologous to *TERMINAL FLOWER 1* (*TFL1*) that suppresses the floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) and maintains the inflorescence meristem in *Arabidopsis*. *MdTFL1* mRNA was expressed preferentially in apple vegetative tissues such as apical buds, stems and roots of seedlings, and expression peaked in early July in apical buds, about two weeks prior to floral bud differentiation. Transgenic *Arabidopsis* expressing *MdTFL1* flowered noticeably later than wild-type plants and exhibited a phenotype similar to that of transgenic *Arabidopsis* overexpressing *TFL1*. These results suggest that *MdTFL1* is involved in the maintenance of the vegetative phase in apple and that it functions analogously to *TFL1*.


*Keywords:* *CETS*; Juvenility; *Malus* × *domestica*; *MdTFL1*; *TFL1*

## 1. Introduction

The development of all woody plants from seed includes a juvenile phase lasting up to 40 years in certain forest trees [1]. During the juvenile phase, flowering does not occur and cannot be induced under normal conditions [1]. Thus, the breeding of fruit trees such as the apple (*Malus* × *domestica* Borkh.) often takes more than 20 years including periods of cross-pollination, seedling selection and regional trials to produce varieties that meet consumer demands. Generally, the juvenile period of *M.* × *domestica* lasts 7–8 years [2], but a certain *Malus* species used as root stock neither flowers nor sets fruit for substantially more than 8 years. Hence, the long juvenile phase is the primary factor that limits the efficient breeding of fruit trees, and thus tree fruit production would benefit from a better understanding of the mechanism of apple flower induction and development. However, the genetic factors that control flower induction in apple trees have yet to be investigated.

The most striking advances in our understanding of the genetic control of flowering time come from studies in *Arabidopsis* [3,4]. Three *Arabidopsis* genes that control flowering time have been isolated, namely *LEAFY* (*LFY*) [5], *APETALA1* (*AP1*) [6] and *TERMINAL FLOWER 1* (*TFL1*) [7,8]. In transgenic *Arabidopsis*, over-expression of *LFY* or *AP1* shortens the juvenile period and causes early flowering [9,10] whereas *TFL1* over-expression causes late flowering [11]. *TFL1* plays a key role in the maintenance of the inflorescence meristem by preventing the expression of *LFY* and *AP1* in the shoot apical meristem [11,12].

Juvenility in fruit trees remains largely undefined. As such, there is keen interest in regulatory genes such as *TFL1*, *Antirrhinum CENTRORADIALIS* (*CEN*) [13] and tomato *SELF-PRUNING* (*SP*) [14], which maintain the vegetative phase and promote the emergence of shoots rather than flowers. There is also strong interest in genes *LFY* and *AP1*, which promote flowering. *CEN*, *TFL1* and *SP* are closely related and belong to a small gene family (*CETS*) that

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encodes ~23-kDa proteins. These three plant genes were the first to be assigned biological functions [15] and their amino acid sequences exhibit similarity with a group of mammalian phosphatidylethanolamine binding proteins (PEPBs) originally named for their ability to bind phospholipids in vitro [16]. *FLOWERING LOCUS T* (*FT*) also belongs to the CETS family and is a homologue of *TFL1* [17,18]. However, *FT* antagonizes the *TFL1* function and thereby promotes flowering in concert with *LFY* [17,18].

In mammals, PEBPs are believed to be precursors of a hippocampal neurostimulatory peptide (HCNP), and PEBPs are inhibitors of Raf-1 kinase activity [19]. The tomato protein SP, a member of the CETS family, interacts with several proteins termed SIPs (SP-interacting proteins) that include SPAK (SP-associated kinase), a NIMA-like kinase, and 14-3-3 isoforms [15]. Tomato SIPs bind to CETS proteins such as *Antirrhinum CEN*, as well as to *Arabidopsis TFL1* and its functional antagonist FT, providing evidence that SP/SIPs interactions in tomato are conserved in distantly related plants [15].

Several types of apple genes that may be involved in flower development have been isolated and characterized. Of the *MADS*-box genes from apple, *MdMADS2* and *MdMADS5* have been analyzed in detail [20–22]. *MdMADS2* and *MdMADS5* promote flowering in transgenic tobacco and *Arabidopsis*, respectively, and they function analogously to *AP1* [21,23]. Additionally, apple *AFL1* and *AFL2*, two orthologues of *LFY*, are involved in flowering. *AFL1* and *AFL2* share 90% homology within their coding regions, and while these genes function similarly to *LFY* their expression patterns differ from that of *LFY* [24].

The genes mentioned above promote flower induction or flower development in apple. However, progression from the juvenile phase in perennial crops such as fruit trees also requires that juvenility/vegetative maintenance factors be cleared. The present work describes the isolation and characterization of a *TFL1*-like gene, termed *MdTFL1*, which is involved in the maintenance of juvenile/vegetative phase in apple.

## 2. Materials and methods

### 2.1. Plant materials

The apple (*Malus* × *domestica* Borkh.) cultivar 'Jonathan' was used to isolate and characterize the *MdTFL1* gene. Apple leaves and flowers were collected from the experimental field at our research center in Morioka, Japan. Jonathan apple seedlings were used for expression analysis. *Arabidopsis thaliana* Columbia (Col) and *tfl1-1* were obtained from the *Arabidopsis* Biological Resource Center at The Ohio State University and wild-type plants were employed for *Agrobacterium*-mediated transformation. *Arabidopsis* seeds were stratified for 3–4 days at 4 °C and then grown on an agar plate containing 0.5 × Murashige and Skoog (MS) medium (Wako Pure Chemicals Co. Ltd., Tokyo, Japan) in growth chambers at 22 °C. Ten days after sowing the agar plate, seedlings were transferred to soil and grown in growth chambers under long-day (LD) conditions (16 h light/8 h dark).

### 2.2. Gene cloning

Full-length *MdTFL1* cDNA was obtained by the 5′ and 3′ rapid amplified cDNA ends (RACE) method [25]. Cassette-ligated cDNAs from the shoot apexes of apples were prepared using the LA-PCR cloning kit (Takara Biomedicals, Tokyo, Japan). Primers used in this study are listed in Table 1. The first amplified apple cDNA contained 233 bp between the 5S and 3A designed from *TFL1*, *CEN* and *SP* cDNA sequences. The 3′ RACE was carried out between

Table 1
Primer sequences used in PCR cloning of *MdTFL1*

| Primer | Oligonucleotide[a] |
|---|---|
| Primers for an internal fragment | |
| 5S sense primer | 5′-ATTGTGACTGACATCCCAGGC-3′ |
| 3A antisense primer | 5′-CG/TT/CTGIGCA/GTTA/GAAA/GAAIAC-3′ |
| Cassette primers | |
| C1 primer | 5′-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3′ |
| C2 primer | 5′-CGTTAGACGCGTAATACGACTCACTATAGGGAGA-3′ |
| 5′ RACE primers | |
| R1A antisense primer | 5′-TTCGAGTGCTGAAGTGATCCCTC-3′ |
| R2A antisense primer | 5′-CAG CGA CAG GAA GAC CCA GGT CA-3′ |
| 3′ RACE primers | |
| R1S sense primer | 5′-CACCACAGATGCCACATTTGGAA-3′ |
| R2S sense primer | 5′-GAGGTGGTGAGTTATGAGAT-3′ |
| *MdTFL1* specific primers | |
| MdTFL2S sense primer | 5′-CTCTTAAAATGAAAAGAGCC-3′ |
| MdTFL2A antisense primer | 5′-TTCTCACATGTCAATAAGTT-3′ |

[a] Abbreviation: I, inosine.

cassette primer C1 or C2 and either 5S, R1S or R2S. A 428-bp DNA fragment amplified with C2/R2S primers was cloned into pBluescript II SK+ (Stratagene, La Jolla, CA, USA). The 5′ RACE was carried out between cassette primer C1 or C2 and either 3A, R1A or R2A. A 469-bp DNA fragment amplified with C2/R1A primers was cloned as indicated for the 3′ RACE and sequenced. Full-length cDNAs were amplified with the sense primer MdTFL2S and antisense primer MdTFL2A. Various fragments containing a 656-bp cDNA were obtained and cloned into pBluescript II SK+ or pUC119 (Takara Biomedicals). Consequently, four pBluescript II SK+ clones (pBMDTFL1-, 2-, 5-, and 12+) and 2 pUC119 clones (pUMDTFL1- and 3+) were obtained. These clones were sequenced completely by the dideoxy method using a Hitachi SQ5500S automated sequencer (Hitachi, Tokyo, Japan).

### 2.3. DNA blot analysis

Genomic DNA was obtained from young Jonathan apple leaves. The DNA (10 μg) was digested individually with *Bam*HI, *Eco*RI, *Hin*dIII, *Nco*I, *Xba*I, or *Xho*I according to the manufacturer's instructions (Takara Biomedicals) and then separated on a 0.8% agarose gel. The DNA bands were transferred to Hybond-N+ (Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridized with a digoxigenin (DIG)-labeled PCR probe encoding the *MdTFL1* gene. Hybridization was performed in a 0.5 M Na–Pi buffer (0.25 M $Na_2HPO_4$, pH 7.2) containing 7% SDS and 1 mM EDTA at 65 °C for 16 h followed by three washes in a 40 mM Na–Pi buffer containing 1% SDS at 65 °C for 20 min. The washes and detection methods were performed according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). The membrane was exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

### 2.4. Expression analysis

RNA was isolated from Jonathan apples harvested at various stages of development. Detection of *MdTFL1*, *AFL1* and *AFL2* by RT-PCR was performed by using the RT-PCR high (Toyobo, Tokyo, Japan). A specific primer (5′-GTGGCATACATTGTAAATA-3′) for *MdTFL1* and random primers for *AFL1* and *AFL2* were used in reverse transcription reaction with 1 μg of total RNA as a template. Then, *MdTFL1*, *AFL1* and *AFL2* specific transcripts were identified using the following primers: a sense primer 2S and an antisense primer 2A for *MdTFL1* (Table 1), a sense primer 6S (5′-CAGAGGGAGCACCCGTTCATTGTGAC-3′) and an antisense primer AFL1R (5′-TTCATT-CAGTGTGCCCTAGCC-3′) for *ALF1* and a sense primer 6S and an antisense primer AFL2R (5′-TCAAACT-CTCTCTGCAGAACTGGC-3′) for *AFL2*. PCR reactions were run for 40 cycles at 50 °C. The PCR products were run on 1.5% (w/v) agarose gel and then blotted on the Hybond-N+ (Amersham Pharmacia Biotech). Hybridizations were

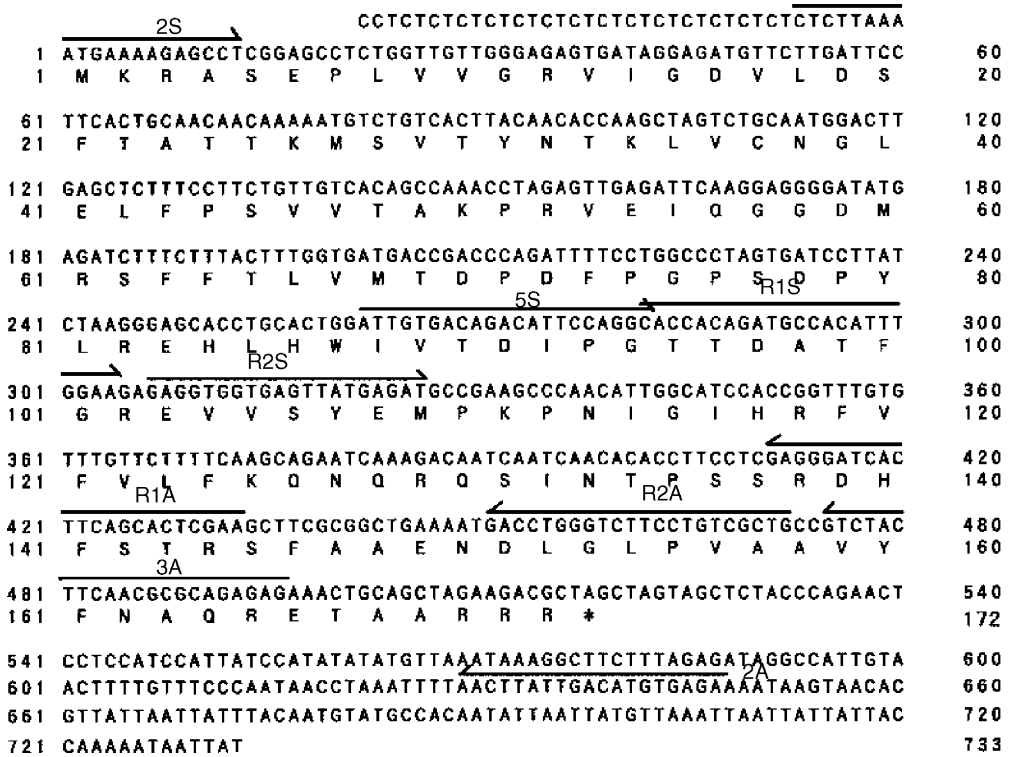
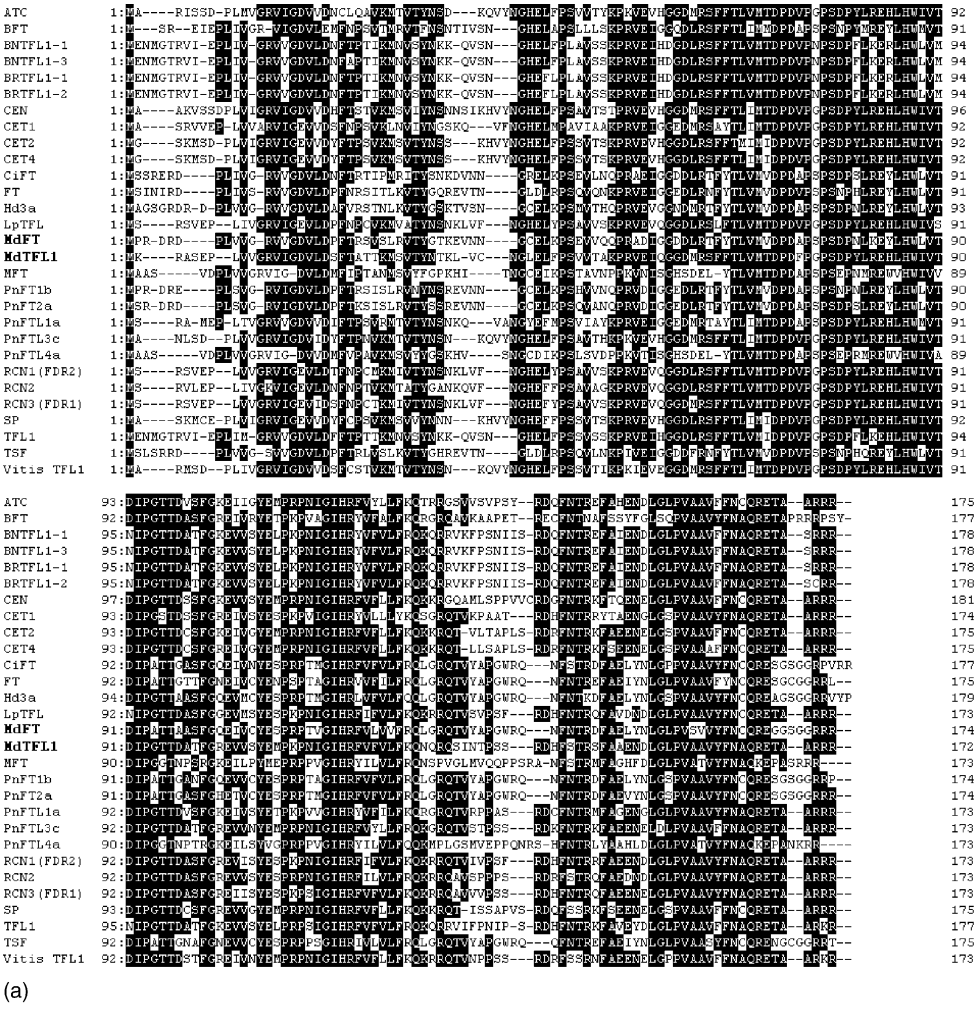
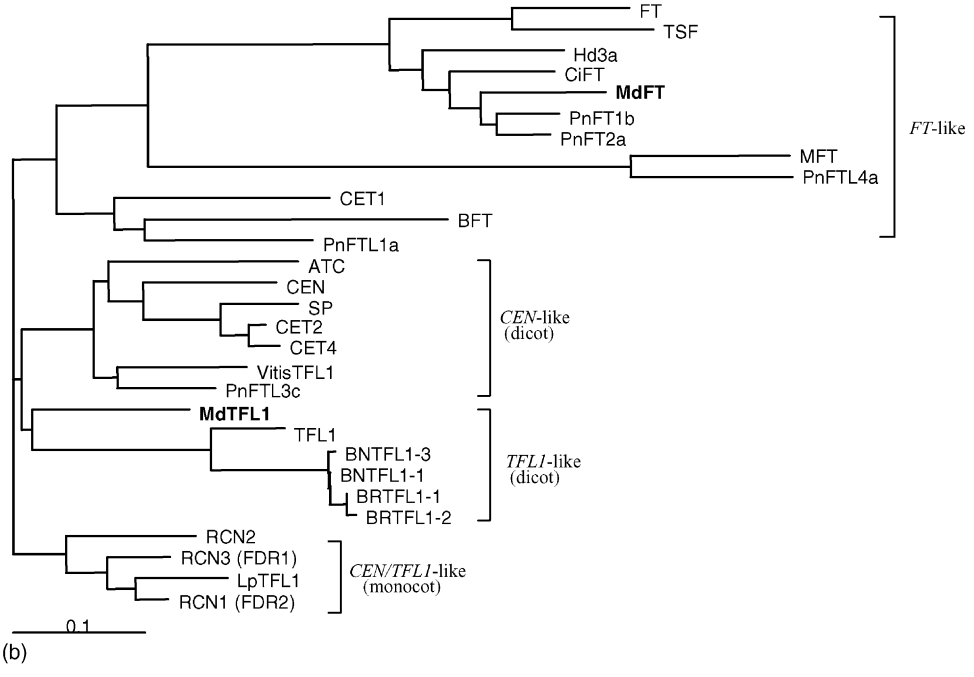



Fig. 1. Nucleotide and deduced amino acid sequences (single-letter code) of *MdTFL1* cDNA. The asterisk (*) indicates a stop codon. Arrows above the sequences indicate the primers used for RACE-PCR.



(a)




(b)

Fig. 2. (a) Comparison of the amino acid sequences of Md*TFL1* and MdFT with those of other CETS proteins. The proteins (translated from the cDNA sequence where necessary) are ATC [28], BFT [18], BNTFL1-1, BNTFL1-3, BRTFL1-1, and BNTFL1-2 [39], CEN (accession no. S81193), CET1, CET2 and CET4 [37], CiFT (accession no. AB027456), FT (accession no. AB027504), Hd3a (accession no. AB052944), and LpTFL1 [33], MdTFL1 and MdFT (accession nos.

performed using DIG-labeled PCR probes specific for *MdTFL1*, *AFL1* or *AFL2*. The detection methods were performed as for DNA blotting using the manufacturer's protocol (Roche Diagnostics). Chemiluminescence was detected using the LAS-1000 image analyzer (Fuji Photo Film).

### 2.5. Construction of transformation vectors

pBMDTFL12+ was cut with *Bam*HI and *Kpn*I to release the *MdTFL1* cDNA fragment, that was then ligated into pUC119 (cut with the same enzymes) yielding pUMDTFL12.1+. pUMDTFL12.1+ was then cut with *Xba*1 and *Sac*I to release the *MdTFL1* cDNA fragment that was subsequently ligated in the sense orientation into the binary vector pSMAK251 containing the CaMV 35S promoter [26] cut with the same restriction enzymes. The resulting plasmid was named pSMDTFL12.1.2+ (35S::*MdTFL1* construct). The *TFL1* cDNA (EST 129D7T7) was obtained from the *Arabidopsis* Biological Resource Center at The Ohio State University. *TFL1* cDNA was amplified by LA-PCR using EST 129D7T7 as a template and cloned into a T-tailed *Sma*I site of pUC119, producing pUTFL129.5. After confirming the *TFL1* sequence using an automated DNA sequencer (Hitachi), pUTFL129.5 was cut with *Xba*I and *Sac*I and the liberated *TFL1* fragment was then ligated into the binary vector pSMAK251 cut with the same restriction enzymes, yielding pSTFL129.5.1 (35S::*TFL1* construct).

### 2.6. Transformation of Arabidopsis

*A. tumefaciens* strain EHA101 was used to transform *Arabidopsis thaliana* (Col) plants by the floral-dip method [27]. For the selection of transformed plants, sterilized seeds were suspended in 0.1% sterile agar, plated on kanamycin selection plates, and then transferred to a growth chamber (BIOTRON, Nippon Medical and Chemical Instruments Co., Ltd., Tokyo, Japan) set at 22 °C under LD conditions. The selection plates contained a 0.5 × MS medium (Wako Pure Chemicals Co., Ltd.), 0.8% agar (Difco Laboratories, Detroit, MI, USA), 500 mg/l cefotaxime (Wako Pure Chemicals Co., Ltd.), and 25 mg/l kanamycin monosulfate (Meiji Seika Kaisha Ltd., Tokyo, Japan). Transformants were identified as kanamycin-resistant when seedlings in the medium produced green leaves and well-established roots. Resistant transformants were transplanted to moistened potting soil composed of vermiculite and perlite [1:1 (v/v)] after 2–5 adult leaves had developed. The day of sowing was counted as day 0. Morphological analyses were performed on the primary ($T_0$) and subsequent generations ($T_1$ and $T_2$).

## 3. Results

### 3.1. Cloning the apple MdTFL1 gene

To investigate the genes that play a role in apple juvenility, we cloned *MdTFL1*, a putative homologue of *Arabidopsis TFL1*. A cDNA library was constructed from apple apical buds to facilitate the isolation of full-length *MdTFL1* cDNA using the RACE method. Primer sites and the nucleotide sequences of primers used for cloning are shown in Fig. 1 and Table 1, respectively.

The cDNA and predicted amino acid sequences for *MdTFL1* are shown in Fig. 1. The *MdTFL1* cDNA coding region exhibits 76%, 73%, 71% and 58% sequence identity to *TFL1*, *CEN*, *SP* and *FT*, respectively. The protein product predicted from the *MdTFL1* sequence comprises 172 residues and exhibits similarity to *TFL1* (75%), *CEN* (74%), *SP* (72%) or *FT* (55%) (Fig. 2a). The gene was therefore designated *MdTFL1* for *Malus domestica TFL1* homologue. A phylogenic comparison of *MdTFL1* with other CETS family members showed that it is grouped with *FT*-like, *CEN*-like (dicot), *TFL1*-like (dicot), and *CEN*/*TFL1*-like (monocot) proteins (Fig. 2b). Apple genomic DNA was digested individually either with *Bam*HI, *Eco*RI, *Hind*III, *Nco*I, *Xba*I or *Xho*I, and DNA blotting was performed using an *MdTFL1* probe. One to four major bands were detected in each digest, suggesting that *MdTFL1* homologue exists in apple (Fig. 3).

### 3.2. Expression pattern of MdTFL1 in apple

*MdTFL1* mRNA expression was analyzed in various living tissues and in apices of current apple shoots during different stages of development by RT-PCR southern blot. Total RNA was isolated from reproductive tissues (sepals, petals, stamens, and carpels) and vegetative tissues (mature leaves and apical buds of vegetative shoots and cotyledons, stems, roots, and apical buds of seedlings). *MdTFL1* mRNA was expressed in vegetative tissues, such as apical buds, seedling stem, and roots, but not in reproductive tissues such as floral organs (Fig. 4a). To elucidate seasonal changes in *MdTFL1* mRNA expression in the apical buds, apices were collected from current apple shoots each month from June to February. Floral buds at the balloon stage were also collected in late April. Total RNA was isolated from each sample and subjected to expression analysis. *MdTFL1* mRNA was expressed strongly in early July, about two weeks before floral bud differentiation with gradual decrease to late-July (Fig. 4b). It was consistent with the result of expression analysis in different tissues that the *MdTFL1* mRNA was detected in vegetative shoots (water sprout) in June (Fig. 4b,

AB052994 and AB161112) and MFT [18], PnFT1b, PnFT2a, and PnFTL1a (accession nos. AB109804, AB110009 and AB161110), RCN1, RCN2, and RCN3 [34], SP (accession no. U84140), TFL1 (accession no. U77674), and TSF [18], and Vitis TFL1 (*Vitis vinifera* TFL1-like protein, accession no. AF378127). Identical residues at each position are shown in black. (b) Phylogenic tree of MdTFL1, MdFT and other CETS family protein sequences in (a). The ClustalW program was used to align 28 complete protein sequences. Bold characters represent apple proteins.

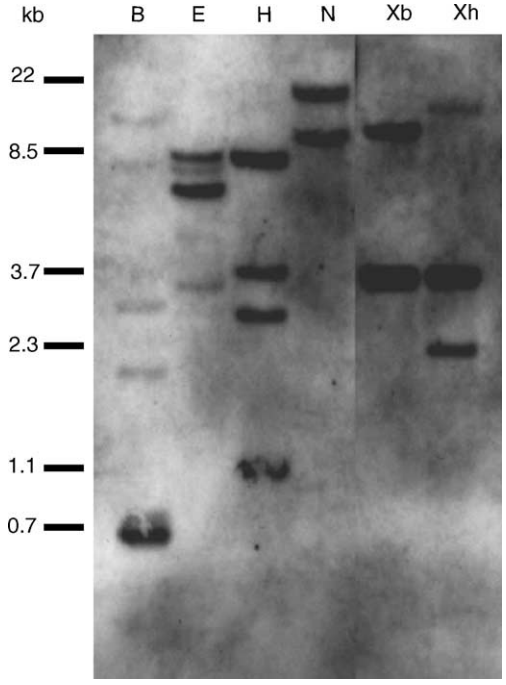



Fig. 3. DNA blot analysis. Genomic DNA was isolated from leaves of the apple cultivar 'Jonathan'. DNA aliquots (10 μg) were digested with individual restriction enzymes and subjected to agarose gel electrophoresis. The DNA blot was hybridized with a DIG-labeled *MdTFL1* DNA probe. Restriction enzymes used to digest apple DNA are shown at the top: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; Xb, *Xba*I; Xh, *Xho*I. Numbers to the left indicate DNA size markers in kbp.

6.19 V). In contrast, the expression of *AFL1*, an apple orthologue of *FLO/LFY* [24], was detected when the expression level of *MdTFL1* was relatively low (Fig. 4b). On the other hand, the expression of *AFL2*, another apple orthologue of *FLO/LFY*, was detected constantly during flower development as previously reported [24].

### 3.3. MdTFL1 transgene delays Arabidopsis flowering

To determine whether the *MdTFL1* gene functions analogously to *Arabidopsis TFL1*, we constructed a binary vector pSMDTFL12.1.2+ containing full-length *MdTFL1* cDNA inserted in the sense-oriented direction under control of the 35S CaMV promoter. pSMDTFL12.1.2+ was introduced into wild-type *Arabidopsis* plants (Col) by *Agrobacterium*-mediated transformation. Thirty-one independent kanamycin-resistant transgenic plants were identified. Five of the 31 primary transformants ($T_0$ generation) exhibited significantly delayed flowering while 7 flowered slightly later than wild-type plants (data not shown).

The $T_1$ generation inherited the phenotype of the $T_0$ generation. Quantitative characteristics of six independent kanamycin-selected segregating $T_2$ transgenic lines are shown in Table 2. The earliest flowering occurred at 29.0 days after sowing in one line, while the latest flowering occurred at 39.2 days compared to 24.3 days for the wild-type control plants under LD photoperiods (Table 2). The number of rosette leaves, a measure of developmental time to flowering, was higher in the 35S::*MdTFL1* transgenic plants (e.g., 14.3 for S10-2 seedlings compared to 9.3 and 6.4 for the wild-type and *tfl1-1* control plants, respectively). At 35 days after sowing, a typical 35S::*MdTFL1* transformant had produced 12-15 rosette leaves but no flower buds or bolting (Fig. 5b). In contrast, the wild-type plants displayed many flowers and brown pods on day-35 (Fig. 5a). In addition, there was little difference between wild-type *Arabidopsis* control plants and those transformed with a 35S::*MdTFL1* antisense construct with respect to days to flowering and number of rosette leaves at flowering (data not shown).

### 3.4. Comparison of 35S::MdTFL1 and 35S::TFL1 transgenic Arabidopsis

35S::*MdTFL1* transformants exhibited a phenotype similar to that of 35S::*TFL1* transformants. One 35S::*MdTFL1* $T_1$-transformant line with a strong phenotype (S10-2; Fig. 5d) did not flower until day-90. The 35S::*TFL1* transformant (Fig. 5c, right side) had not yet flowered whereas the wild-type control plant (Fig. 5c, left side) had flowered and set fruits at 42 days after sowing. Each of these transformants displayed long primary inflorescences, the uppermost 5–6 of which displayed normal flower buds in appearance (Fig. 5e,f). Floral buds formed in the 35S::*MdTFL1* and 35S::*TFL1* transformants often failed to flower or set seeds. In 35S::*MdTFL1* and 35S: *TFL1* transformants, the number of cauline leaves at flowering increased relative to that of wild-type control plants (Table 2).

## 4. Discussion

Apple *MdTFL1* exhibits a high degree of sequence homology to *TFL1*, a member of the CETS family. A comparison of *MdTFL1* with other CETS family members shows that it groups with *TFL1* and four *Brassica* proteins (Fig. 2b). It is logical that *MdTFL1* groups with proteins from dicots rather than monocots because the apple belongs to Rosaceae family, which consists of dicotyledonous plants. Recent studies have revealed that *CEN/TFL1/SP* and *FT* are the members of a small gene family. There are six members in *Arabidopsis* and approximately six in tomato [28,29]. In pea, two *TFL1*-like genes exist and function differently [30]. Poplar, which is a woody plant, such as the apple, contains at least eight *CETS* genes in its genome (T. Igasaki, personal communication). The DNA blot analysis for *MdTFL1* implied the existence of other *TFL1*-like gene(s) in apple. Recently, an apple gene *MdFT* (GenBank Accession no. AB161112) homologous to *FT*, which will be a member of CETS family in apple, was isolated. Several regions of notable sequence homology have been described for mammalian PEBPs. They include a D-P-D-x-P motif

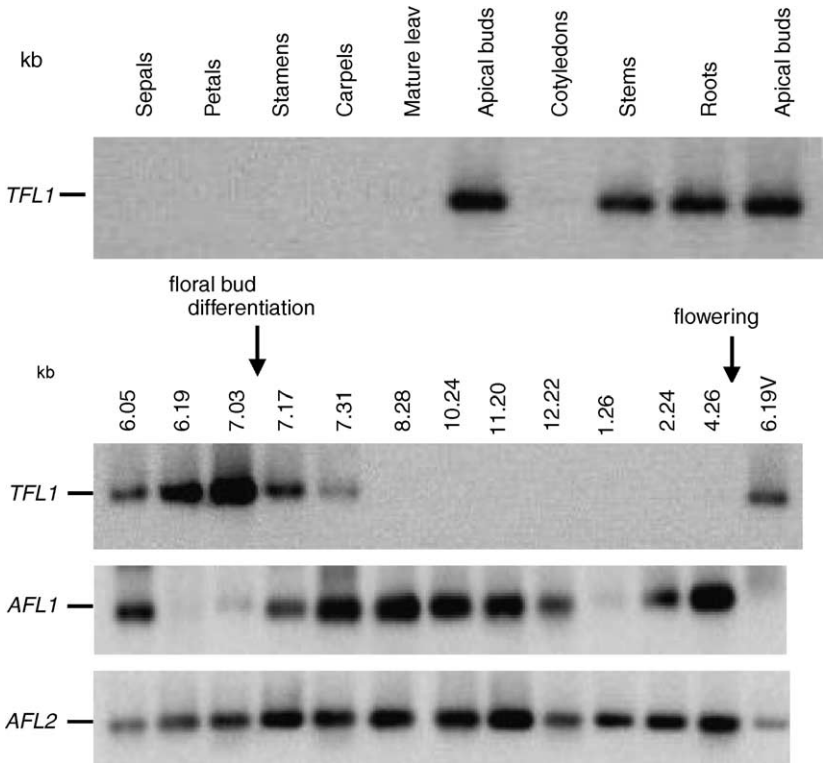



Fig. 4. RT-PCR analysis. 1 μg of total RNA was used for RT-PCR. Subsequent southern blots were performed using DIG-labeled DNA probes for *MdTFL1*, *AFL1* and *AFL2*. (a) PCR for *MdTFL1* was performed on cDNAs obtained from reproductive and vegetative tissues, then PCR products were hybridized with a DIG-labeled *MdTFL1* DNA probe. Reproductive tissues: sepals, petals, stamens, and carpels. Vegetative tissues: apical bud (V), cotyledons, stems, roots, and apical buds. V, vegetative shoots. (b) PCRs for *MdTFL1*, *AFL1* and *AFL2* were performed on cDNAs obtained from apical buds of apple during flower development, then PCR products were hybridized with DIG-labeled *MdTFL1*, *AFL1* and *AFL2* DNA probes, respectively. Numbers above the lanes show the date of harvest (month, day). Arrows indicate the onset of floral bud differentiation and flowering. EtBr, ethidium bromide.

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 [31]. In *MdTFL1*, a D-P-D-x-P motif runs from residue 70 to 74, a histidine residue is positioned at 86, and a G-x-H-R motif runs from residue 115 to 118. These motifs are conserved in other members of the CETS family.

*MdTFL1* mRNA was expressed in apical buds of vegetative shoots (water sprout) and seedling stems, roots and apical buds, but not in sepals, petals, stamens, or carpels. These observations demonstrate that *MdTFL1* is expressed preferentially in vegetative tissues, although *MdTFL1* is not expressed in mature leaves like *TFL1* in *Arabidopsis* [7,32]. In *Antirrhinum*, however, *CEN* mRNA is present not only in the apical meristem but in other tissues [13], and tomato *SP* is expressed throughout development in all the primordial organs [14]. Unlike *MdTFL1*, other *CETS* family genes such as *LpTFL1* in ryegrass, *RCN2* in rice and *PsTFL1* genes in

Table 2
Phenotype of $T_2$-transformed *Arabidopsis* expressing the *MdTFL1*

| Controls and transgenic lines[a] | Days to flowering[b,d] | Rosette leaves at flowering[c,d] | Cauline leaves at flowering[c,d] | No. of plants |
|---|---|---|---|---|
| wt | 24.3 ± 0.9 | 9.3 ± 0.9 | 2.1 ± 0.3 | 10 |
| *tfl1-1* | 20.4 ± 1.1 | 6.4 ± 0.8 | 1.1 ± 0.3 | 9 |
| 35S::*TFL1* | 34.3 ± 5.0 | 15.3 ± 2.9 | 5.0 ± 1.6 | 3 |
| S6-5 | 29.0 ± 3.4 | 11.7 ± 2.1 | 3.7 ± 1.3 | 6 |
| S10-2 | 33.5 ± 2.2 | 14.3 ± 1.5 | 5.5 ± 0.9 | 8 |
| S21-2 | 36.1 ± 3.7 | 13.5 ± 1.9 | 6.3 ± 1.3 | 8 |
| S22-2 | 39.2 ± 7.7 | 13.8 ± 2.0 | 5.8 ± 2.3 | 6 |
| S28-2 | 36.6 ± 4.6 | 12.4 ± 2.2 | 7.2 ± 2.3 | 5 |

[a] Seedlings from controls (wt, *tfl1-1*, 35S::*TFL1*) and secondary transformants ($T_1$) carrying the *MdTFL1* gene, were grown under long-day (16 h light/8 h dark) conditions. $T_2$-seedlings were selected with kanamycin.

[b] Days to flowering indicates the time at which flower primordia first became visible to the naked eye.

[c] Rosette and cauline leaves were counted on the day flower primordia became visible.

[d] All values are mean ± S.E.M.

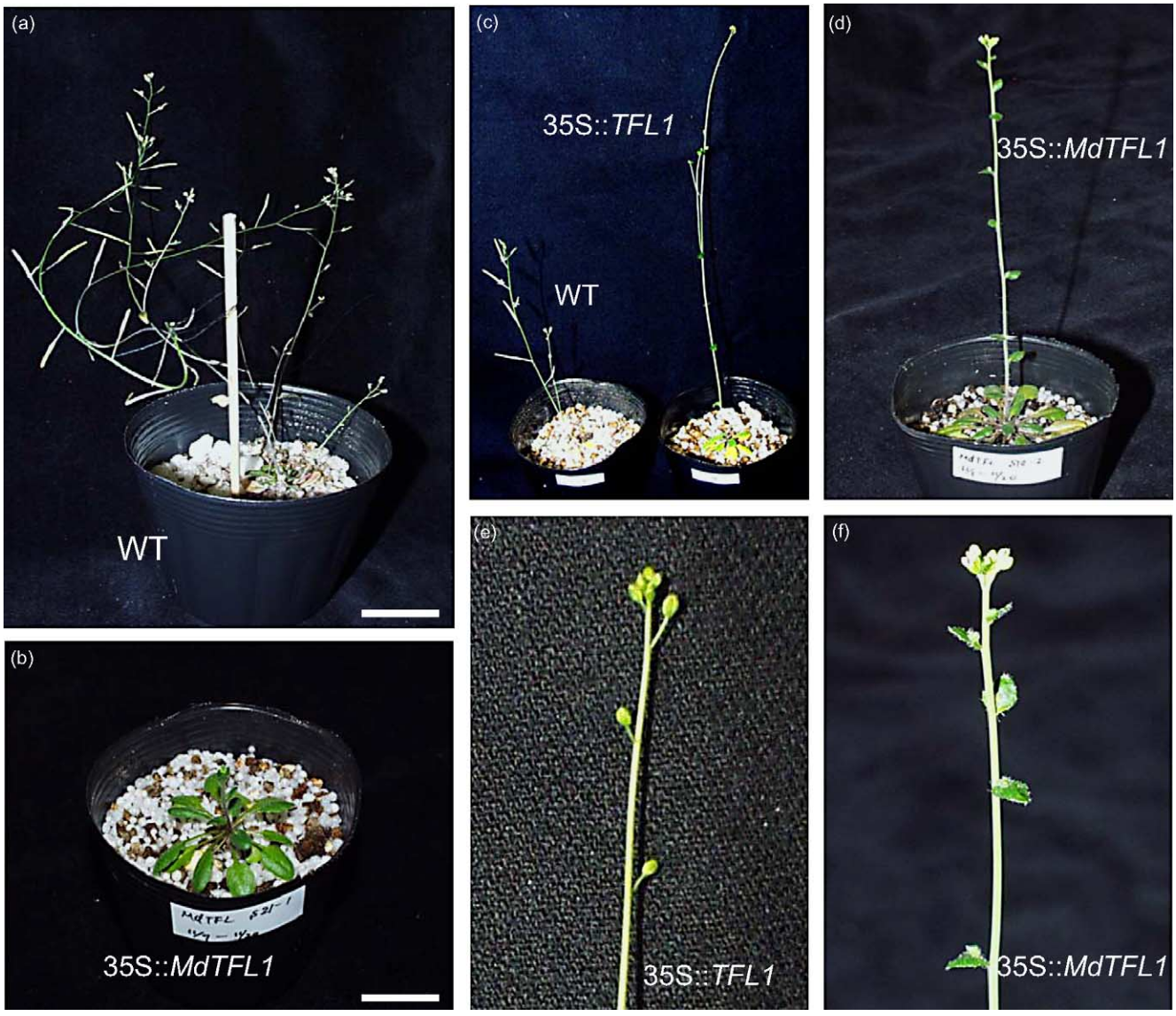



Fig. 5. Comparison of 35S::*MdTFL1* and 35S::*TFL1* transgenic *Arabidopsis* plants: (a) and (b) 35-day-old plant (ecotype Columbia) grown under LD conditions (16 h light/8 h dark). (a) Wild-type (WT) control plant. (b) $T_1$ transgenic line S21-1. No flower buds were present in line S21-1, although brown siliques were developing in the control plant. (c) Wild-type (WT) control plant (left) and 35S::*TFL1* transformant (right) at 42 days after sowing. (d) 35S::*MdTFL1* transformant (line S10-2) at 90 days after sowing, when a flower first opened. (e) Inflorescence of a 35S::*TFL1* transformant. (f) Inflorescence of a 35S::*MdTFL1* transformant. Scale bars, 3.0 cm.

pea are expressed in both vegetative and reproductive tissues [30,33,34]. *MdTFL1* expression differed from that of *SP*, *LpTFL1*, *RCN2* and *PsTFL1* in that *MdTFL1* is expressed preferentially in vegetative tissues. The expression pattern of *MdTFL1* in different tissues resembled that of *SP9D*, a member of the CETS family in tomato since both genes were expressed in shoot apices and roots, not in reproductive tissues [29]. In fact, *MdTFL1* exhibits greater similarity to *SP9D* (77%) than to *SP* (72%) in amino acids sequences.

Regarding seasonal-dependent *MdTFL1* expression in the apices of apple, the mRNA was expressed strongly in early July (about 8 weeks after full bloom), approximately two weeks prior to the initiation of floral bud formation. Thereafter, expression decreased gradually to late July (Fig. 4b). *MdTFL1* is possibly involved in the regulation of flower induction from late June to early July (6–8 WAFB) since this period is thought to be critical for the determination of meristem identity in apple [20,35,36]. In *Arabidopsis*, *Antirrhinum* and tobacco, the production of flowers or shoots most likely depends on the relative expression patterns of *TFL1*/*CEN*-like genes and floral meristem identity genes such as *LFY* and *AP1* [32,37]. Interestingly, the seasonal expression level of *MdTFL1* in apple apices appears to be complementary to that of *AFL1*, which gradually increases from early July to late August, although *AFL2* expression is constant during flower development (Fig. 4b). Thus, the induction of flowering may also depend on the relative expression of *MdTFL1* and *AFL1* in apple. The genetic interaction of *MdTFL1* and *AFL1*

remains to be analyzed to understand the regulation of flower induction in apple.

Over-expression of *MdTFL1* retarded the transition from the vegetative to the reproductive phase in transgenic *Arabidopsis* plants (Fig. 5 and Table 2). 35SS::*MdTFL1* transformants exhibited increased numbers of rosette and cauline leaves and an extended vegetative phase compared to wild-type control plants. These results suggest that *MdTFL1* maintains the inflorescence meristem in transgenic *Arabidopsis*, resulting in a delay of flowering. In addition, 35S::*MdTFL1* transformants resembled 35S::*TFL1* transformants not only in delayed flowering but also in morphological characteristics, which may be related to the similar expression pattern between *MdTFL1* and *TFL1* (Figs. 4 and 5).

In woody plants such as apple, the maintenance of the juvenile phase is one of the most important early-stage events during plant development. In apple, however, genes that control the transition from the vegetative to the reproductive phase have not yet been determined. Our work shows that *MdTFL1* is a member of the CETS family of apple and that over-expression of *MdTFL1* causes delayed flowering in transgenic *Arabidopsis*. Analysis of the *MdTFL1* sequence, expression pattern and function suggests that this gene may play a key role in maintaining the juvenile and/or vegetative phase in apple. Recently, we confirmed that several transgenic apples with antisense *MdTFL1* flowered extremely early [38]. Future transgenic approaches may suppress the expression of endogenous *MdTFL1* so as to reduce the generation time of apple trees that normally exhibit a long juvenile period.

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