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Molecular Characterization of *FLOWERING LOCUS T*-Like Genes of Apple (*Malus×domestica* Borkh.)

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The two *FLOWERING LOCUS T* (*FT*)-like genes of apple (*Malus×domestica* Borkh.), *MdFT1* and *MdFT2*, have been isolated and characterized. *MdFT1* and *MdFT2* were mapped, respectively, on distinct linkage groups (LGs) with partial homoeology, LG 12 and LG 4. The expression pattern of *MdFT1* and *MdFT2* differed in that *MdFT1* was expressed mainly in apical buds of fruit-bearing shoots in the adult phase, with little expression in the juvenile tissues, whereas *MdFT2* was expressed mainly in reproductive organs, including flower buds and young fruit. On the other hand, both genes had the potential to induce early flowering since transgenic Arabidopsis, which ectopically expressed *MdFT1* or *MdFT2*, flowered earlier than wild-type plants. Furthermore, overexpression of *MdFT1* conferred precocious flowering in apple, with altered expression of other endogenous genes, such as *MdMADS12*. These results suggest that *MdFT1* could function to promote flowering by altering the expression of those genes and that, at least, other genes may play an important role as well in the regulation of flowering in apple. The long juvenile period of fruit trees prevents early cropping and efficient breeding. Our findings will be useful information to unveil the molecular mechanism of flowering and to develop methods to shorten the juvenile period in various fruit trees, including apple.

Keywords: Apple • *FLOWERING LOCUS T* (*FT*) • Flowering time • Juvenility • *Malus×domestica* Borkh.

Abbreviations: *AP1*, *APETALA1*; *ATC*, *ARABIDOPSIS THALIANA CENTRORADIALIS*; *BFT*, *BROTHER OF FT AND TFL1*; CaMV, cauliflower mosaic virus; CAPS, cleaved amplified polymorphic sequence; *CEN*, *CENTRORADIALIS*; DAF, days after flowering; DIG, digoxigenin; FBS, fruit-bearing shoots; *FLC*, *FLOWERING LOCUS C*; *FT*, *FLOWERING LOCUS T*; *FUL*, *FRUITFULL*; LD, long day; *LFY*, *LEAFY*; LG, linkage group; *MFT*, *MOTHER OF FT AND TFL1*; N–J, Neighbor–Joining; RT–PCR, reverse transcription–PCR; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*; SS, succulent shoots; SSR, simple sequence repeat; *TFL1*, *TERMINAL FLOWER1*; *TSF*, *TWIN SISTER OF FT*.

The nucleotide sequences reported in this paper have been submitted to the DDBJ under accession numbers AB458504 (*MdFT2* mRNA), AB458505 (*MdFT2* genomic DNA), AB458506 (*MdFT1* genomic DNA), AB501124 (*MdSOC1a* mRNA), and AB501125 (*MdSOC1b* mRNA).

Introduction

Apple (*Malus* spp.), which belongs to the Rosaceae subfamily Maloideae, is one of the most important fruit tree crops in the world. However, the long juvenile phase characteristic of woody plants, including apple, makes their breeding cycle slower (Zimmerman 1972, Hackett 1985). In apple, the juvenile phase generally lasts from 4 to 8 years or more. For example, the 'Fuji' apple (*Malus×domestica* Borkh.) from 'Ralls Janet'×'Delicious',

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one of the most popular apple cultivars in the world (O'Rourke et al. 2003), set fruit for the first time 12 years after sowing (Sadamori et al. 1963), whereas the 'Sansa' apple from 'Gala'×'Akane', top-grafted on fruit-bearing trees for early selection, set fruit after 7 years (Yoshida et al. 1988). In general, selection efficiency is limited until the seedlings set fruit. In the case of fruit breeding, the most important traits for selection, such as texture, flavor, ripening time and shelf life, are related to the fruit itself. Therefore, various practical techniques to accelerate flowering and fruiting of seedlings in the juvenile phase have been considered for years. In apple, grafting the seedling onto fruit-bearing trees (top grafting) or onto dwarfing rootstocks, such as 'Malling 9 (M. 9)' (Pearl 1932) and 'JM1' (Soejima et al. 1998), results in earlier flowering by 1 or 2 years. After the end of the juvenile phase, flower induction occurs in late June, and flower initiation with morphological changes at the apical meristem starts to form floral primordia in mid July in Morioka, Japan (Kotoda et al. 2000). However, little is known about the physiological and genetic factors involving the transition to flowering in apple.

On the other hand, studies on Arabidopsis (*Arabidopsis thaliana*) and snapdragon (*Antirrhinum majus*) have led to the identification of many genes involved in flowering. In Arabidopsis, the transition from the vegetative to the reproductive phase is initiated by four independent pathways of signal transduction, i.e. the autonomous, and gibberellin-, vernalization- and light-dependent pathways (for reviews, see Koornneef 1998, Levy and Dean 1998). These signals are transmitted via integrator genes, such as *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*)/*AGAMOUS LIKE20* and *FLOWERING LOCUS C* (*FLC*), to the floral meristem identity genes *APETALA1* (*AP1*) and *LEAFY* (*LFY*) at the apical meristems (for reviews, see Araki 2001, Jack 2004, Michaels 2009). In addition to the integrator genes, *TERMINAL FLOWER1* (*TFL1*; Bradley et al. 1997, Oshima et al. 1997) is also a key gene that represses flowering and maintains the inflorescence meristem by preventing the expression of *AP1* and *LFY* (Ratcliffe et al. 1998, Ratcliffe et al. 1999). *TFL1* was the first member of the *TFL1*/*FT* family to be identified as a homolog of an animal phosphatidylethanolamine-binding protein (PEBP), considered to be a Raf-1 kinase inhibitor (Yeung et al. 1999). Thereafter, the Arabidopsis genome was found to contain six members of the *TFL1*/*FT* family, including *ARABIDOPSIS THALIANA CENTRORADIALIS* (*ATC*), *BROTHER OF FT AND TFL1* (*BFT*), *FT*, *MOTHER OF FT AND TFL1* (*MFT*) and *TWIN SISTER OF FT* (*TSF*) (Kardailsky et al. 1999, Kobayashi et al. 1999, Mimida et al. 2001, Yoo et al. 2004, Yamaguchi et al. 2005).

FT was identified as a causative gene for a typical late-flowering mutant *ft*, the phenotype of which was opposite to that of *tfl1* (Kardailsky et al. 1999, Kobayashi et al. 1999). Hanzawa et al. (2005) reported that the antagonistic functions of a floral activator and a repressor encoded by *FT* and *TFL1*, respectively, could be converted by a single amino acid substitution. Recently, it was found that the *FT* protein moves from the leaf to the shoot apex, where FT interacts with the bZIP transcription factor, FD, to activate *AP1* in Arabidopsis (Abe et al. 2005, Wigge et al. 2005, Corbesier et al. 2007). In rice (*Oryza sativa*), it was demonstrated that the protein encoded by *Hd3a* (Kojima et al. 2002), a rice equivalent of the *FT* gene, similarly travels from the leaf to the shoot apex (Tamaki et al. 2007), probably in the absence of GF14c (a G-box factor 14-3-3c protein), a direct inhibitor of Hd3a (Purwestri et al. 2009). Furthermore, grafting experiments in cucurbit species (*Cucurbita maxima* and *C. moschata*) demonstrated that the FT protein, but not *FT* mRNA crossed the graft union in the phloem translocation stream (Lin et al. 2007). Experiments in Arabidopsis also revealed that the FT protein is graft transmissible (Notaguchi et al. 2008). The results of these studies suggest that the FT protein acts as a mobile flower-inducing signal, such as a florigen, as noted by Chailahyan (for reviews, see Chailahyan 1968, Giakountis and Coupland 2008, Zeevaart 2008).

Based on studies of Arabidopsis and other plant species, much effort has been devoted to unveiling the molecular mechanism of flowering and to manipulate the flowering time in horticultural trees since the last decade (for reviews, see Benlloch et al. 2007, Hanke et al. 2007, Wilkie et al. 2008). Apple genes related to flowering have been investigated by analyzing its expression patterns (Yao et al. 1999, Kotoda et al. 2000, van der Linden et al. 2002, Esumi et al. 2005, Hättasch et al. 2008) and heterologous transgenic plants (Sung et al. 1999, Kotoda et al. 2002, Wada et al. 2002). In this context, we have searched for the genes that maintain the juvenile/vegetative growth or induce the transition to flowering in apple. As a result, transgenic apple expressing the antisense RNA of *MdTFL1* (an apple ortholog of *TFL1*) showed precocious flowering (Kotoda et al. 2003). Therefore, we further investigated the genes of the *TFL1*/*FT* family as candidates that can shorten the juvenile/vegetative growth phase and manipulate the flowering time in apple (Kotoda and Wada 2005, Kotoda et al. 2006, Mimida et al. 2009). In this study, we have isolated and characterized the two *FT*-like genes of apple and examined their function using transgenic Arabidopsis. Furthermore, we investigated transgenic apple overexpressing the *FT*-like gene to clarify its function in apple.

Results

Identification of *FT*-like genes from apple

We previously isolated *MdFT*, an apple homolog of *FT*, and classified it as an *FT*-like gene by phylogenetic analysis (Kotoda and Wada 2005). However, the result of DNA blot analysis suggested the existence of another *MdFT*-like gene in apple (**Fig. 1A**). Therefore, we screened the cDNA library derived from flower buds of 'Fuji' apple to obtain *FT*-like genes homologous to *MdFT*. As a result, positive clones converged to two kinds of cDNA. One corresponded to *MdFT*, which we renamed *MdFT1*, but the other seemed to be a novel gene homologous

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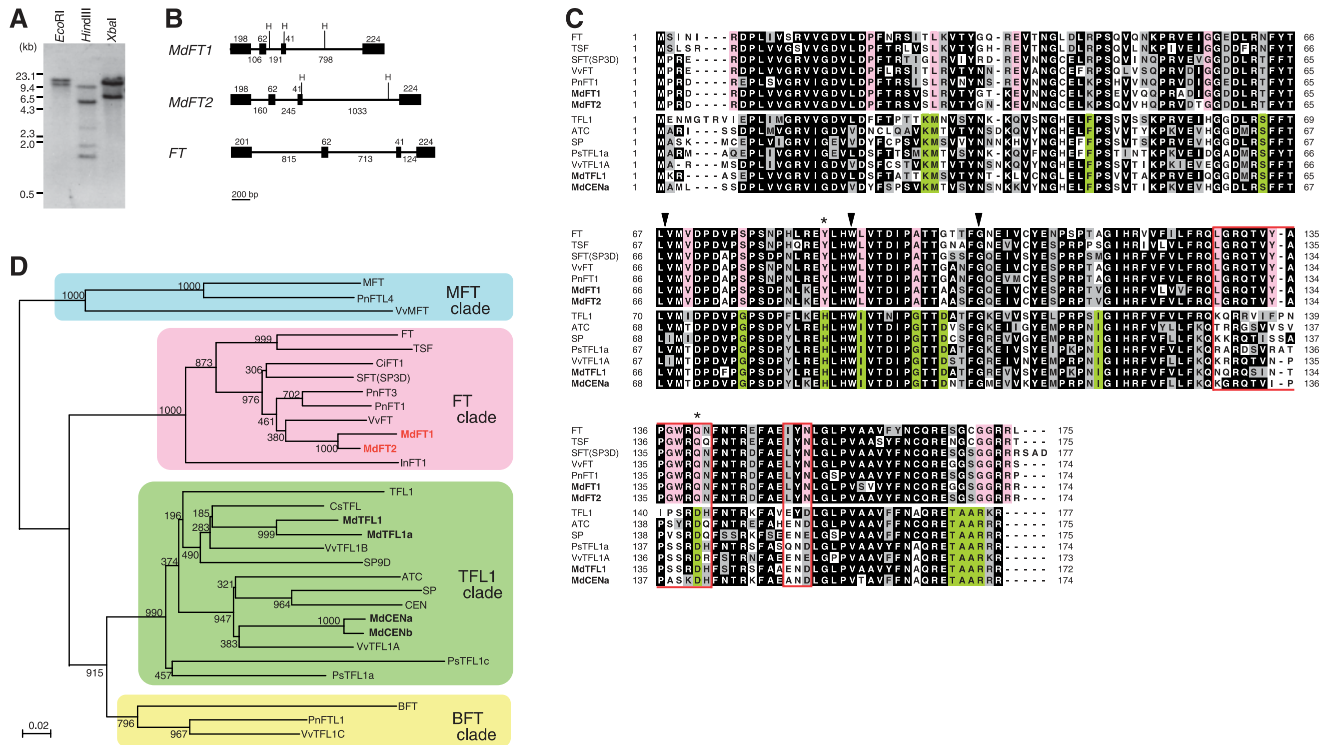

Fig. 1 (A) DNA blot analysis for *FT*-like genes of apple. The genomic DNA (15 µg) of the 'Fuji' apple was digested individually with *Eco*RI, *Hin*dIII or *Xba*I and then separated on a 0.8% (w/v) agarose gel. DNA bands were transferred to Hybond N+ and hybridized with a digoxigenin (DIG)-labeled *MdFT1* cDNA. Hybridization was performed in DIG Easy Hyb (Roche Diagnostics) at 42°C for 16 h followed by two washes in 0.5×SSC containing 1% (w/v) SDS at 68°C for 20 min. The molecular size markers are shown in kb on the left. (B) Schematic representation of the genomic organization of *FT*-like genes of the apple and Arabidopsis *FT* gene. Boxes represent exons and lines represent introns. Numbers represent the lengths (bp) of exons (above the boxes) and introns (below the lines). The scale bar on the map represents approximately 200 bp. H represents a restriction enzyme site of *Hin*dIII in *MdFT1* and *MdFT2* genes. (C) Comparison of the deduced protein sequence of *MdFT1* and *MdFT2* (accession Nos. AB161112 and AB458504, respectively) with those of the *TFL1/FT* family from apple, Arabidopsis, tomato, grapevine, Lombardy poplar and pea. The AGI (Arabidopsis Genome Initiative; http://www.arabidopsis.org) code or accession number of each gene, as pertinent, is as follows: *FT* (AGI code At1g65480), *TSF* (AGI code At4g20370), *SFT* (tomato, accession No. AY186735), *VvFT* (grapevine, accession No. DQ871590), *PnFT1* (Lombardy poplar, accession No. AB106111), *TFL1* (AGI code At5g03840), *ATC* (AGI code At2g27550), *SP* (tomato, accession No. U84140), *PsTFL1a* (pea, accession No. AY340579), *VvTFL1A* (*VvTFL1*) (grapevine, accession No. DQ871591), *MdTFL1* (apple, accession No. AB052994) and *MdCENa* (apple, accession No. AB366641). Amino acids in black and in gray are identical and similar, respectively, in at least seven of the 14 members of the TFL1/FT family. Amino acids common to genes in the FT clade are shown in pink and those in the TFL1 clade are shown in green. The gaps indicated by dashes are attributed to the lack of amino acids. The asterisks indicate the amino acid positions related to antagonistic functions between TFL1 and FT (Hanzawa et al. 2005, Ahn et al. 2006). The first square in red, called segment B, represents the region of a potential ligand-binding pocket in TFL1/FT family proteins, and the second square in red represents a region more conserved in FT than in TFL1 (Ahn et al. 2006). The triangles show the intron positions, which are conserved in the TFL1/FT family proteins. (D) Phylogenetic analysis of TFL1/FT family proteins. The tree was constructed by the Neighbor–Joining (N–J) method for the deduced amino acid sequence of the members of the *TFL1/FT* family from apple (*MdFT1*, *MdFT2*, *MdTFL1*, *MdTFL1a*, accession No. AB366643; *MdCENa* and *MdCENb*, accession No. AB366642), Arabidopsis (*ATC*, *BFT*, *FT*, *MFT*, *TFL1* and *TSF*), grapevine [*VvFT* and *VvMFT*, accession No. DQ871594; *VvTFL1A* (*VvTFL1*) and *VvTFL1B*, accession No. DQ871592; *VvTFL1C*, accession No. DQ871593], Lombardy poplar (*PnFT1* and *PnFT3*, accession No. AB110612; *PnFTL1*, accession No. AB369067; *PnFTL4*, accession No. AB181241), morning glory (*InFT1*, accession No. ABW73562), orange (*CsTFL*, accession No. AY344244), pea (*PsTFL1a* and *PsTFL1c*, accession No. AY343326), Satsuma mandarin (*CiFT1*, accession No. AB027456), snapdragon [*CENTRORADIALIS* (*CEN*), accession No. S81193) and tomato (*SP*, *SFT* and *SP9D*, accession No. AY186738). The protein sequence data were obtained from the DNA database (DDBJ/EMBL/GenBank). The N–J unrooted dendrograms were generated from the alignment of deduced amino acids with the Clustal X program, and the phylogenetic tree was displayed using the N–J plot unrooted program (Perrière and Gouy, 1996). Bootstrap values for 1,000 resamplings are shown in each branch. The unit for the scale bar displays branch lengths (0.02 substitutions/site).

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to *MdFT*, which we designated *MdFT2*. The *MdFT1* and *MdFT2* genes consisted of four exons of 198, 62, 41 and 224 bp encoding a putative protein of 174 amino acids, which resembled the genomic structure of the *FT* gene (**Fig. 1B**). The coding sequence of *MdFT1* cDNA exhibited 94.2% identity to that of *MdFT2* cDNA at the nucleotide level, although the sequence of each exon showed slightly different identities for *MdFT1* and *MdFT2* (93.4, 100.0, 95.1 and 95.1% for the first, second, third and fourth exons, respectively). The genomic sequence including the coding region of *MdFT2* (1,963 bp) was longer than that of *MdFT1* (1,620 bp) due to the extended introns of *MdFT2* (**Fig. 1B**).

Sequence analysis using the deduced amino acid sequences of *TFL1*/*FT* homologs revealed that MdFT1 and MdFT2 were the most similar to each other (94.3% identity), and they also had a high identity with FT-like members, such as VvFT, PnFT1, PnFT3, SFT, CiFT1, TSF and FT (86.7, 85.5, 85.1, 84.5, 83.6, 76.3 and 74.6%, respectively) at the amino acid level (for accession numbers, see the legend of **Fig. 1C**). The alignment of the deduced amino acid sequences of the TFL1/FT family showed that there were 19 amino acid residues specific to FT-like members and 15 residues specific to TFL1/CEN-like members (**Fig. 1C**). In particular, both MdFT1 and MdFT2 had Tyr84 and Gln139 in the positions corresponding to Tyr85 and Gln140 of Arabidopsis FT (**Fig. 1C**, see asterisks). Tyr85/His88 and Gln140/Asp144 are likely to be the most critical residues for distinguishing FT and TFL1 in Arabidopsis (Hanzawa et al. 2005, Ahn et al. 2006).

In order to clarify the relationships among the TFL1/FT family members, we constructed a phylogenetic tree by Neighbor–Joining (N–J) distance analysis of apple, Arabidopsis, grapevine (*Vitis vinifera*), morning glory (*Ipomoea nil*), orange (*Citrus sinensis*), pea (*Pisum sativum*), Lombardy poplar (*Populus nigra*), Satsuma mandarin (*Citrus unshiu*), snapdragon and tomato (*Solanum esculentum*) as shown in **Fig. 1D**. The phylogenetic tree was divided into four major clades, represented by BFT, FT, MFT and TFL1. As expected, MdFT2, together with MdFT1, was classified into the FT clade. FT-like members of apple were more closely related to those of deciduous woody plants, such as grapevine and poplar.

Localization of *MdFT1* and *MdFT2* on a linkage map

The Rosaceae subfamily Maloideae, which includes the genus *Malus*, has a higher haploid base chromosome number, $x = 17$, than other members of the family Rosaceae, probably due to the hypothesized polyploid origin (Sax 1933, Chevreau et al. 1985). Since the DNA blot and sequence analysis suggested that *MdFT1* and *MdFT2* were not allelic (**Fig. 1A**), we attempted to show clearly on which linkage group *MdFT1* and *MdFT2* were located and to consider the evolutionary origin of those genes. Therefore, genetic mapping was performed for the genes using two kinds of F_1 mapping populations from crosses between 'Delicious' (De) and Mitsubakaido (*Malus sieboldii* Rehder) and between 'Ralls Janet' (Ra) and Mitsubakaido (Igarashi et al. 2008). As a result, *MdFT1* was assigned to the south end of linkage group 12 (LG 12) of 'Delicious' (De12), with 70.7 cM from the markers rD05A20–0.9 and E35M48–350 at the north end, whereas *MdFT2* was assigned to the locus close to the marker E38M61–395 at the south end of LG 4 of 'Ralls Janet' (Ra04) (**Fig. 2**).

Expression patterns of *MdFT1* and *MdFT2* in apple

To identify when and where *MdFT1* and *MdFT2* were expressed, quantitative real-time reverse transcription–PCR (qRT–PCR) was performed on various tissues of apple in the adult phase, juvenile phase or tissue culture using primers specific to each gene (**Supplementary Table S1**). The transcript of *MdFT1* accumulated mainly in apical buds of fruit-bearing shoots (FBS) (AB1, on June 24; AB2, on August 7), flower buds (at the balloon stage), floral organs, such as stamens, and whole young fruits. The level of *MdFT1* transcript was very low in the tissues of 1-month-old seedlings in the juvenile phase, such as roots, stems, mature leaves and apical buds of vegetative shoots, with little detection in seeds and cultured shoots, which included apical buds, stems and leaves. The level of *MdFT1* transcript in the mature leaves was relatively low in both the juvenile and the adult phase as compared with that detected in the apical buds and reproductive organs in the adult phase, although the expression of *MdFT1* in mature leaves was higher in the adult phase than in the juvenile phase. The *MdFT1* transcript level in mature fruit and peel was below the detection limit (**Fig. 3A**). On the other hand, the transcript of *MdFT2* was detected mainly in reproductive organs, such as flower buds, sepals, petals, stamens, carpels, receptacles, peduncles and whole young fruits, with some expression also detected in mature fruit (**Fig. 3B**). In apical buds of FBS, we also detected the expression of *MdFT2*, but the expression level of *MdFT2* was relatively lower than that of *MdFT1*. In the tissues of 1-month-old seedlings, *MdFT2* was expressed at relatively higher levels than *MdFT1*; however, the transcript of *MdFT2* in mature leaves was low in both the juvenile and the adult phases, similarly to *MdFT1*. We could not detect the expression of *MdFT2* in seeds and peel (**Fig. 3B**). The transcripts of *MdFT2* accumulated in shoots of cultured tissues more than those of *MdFT1*, although the transcription level of both was much lower than that in the apical buds of FBS.

Seasonal expression pattern in apical buds of apple in the adult phase showed that *MdFT1* was expressed to higher levels than *MdFT2* in FBS from late June to August. In addition, *MdFT1* was expressed relatively more highly in FBS than in succulent shoots (SS), which are vegetative shoots that do not produce flower buds, while the expression level of *MdFT2* was similar between FBS and SS, from early June to mid August (**Fig. 3C, D**). The expression of *MdFT2* peaked in mid September and thereafter decreased during the dormant period with a rapid increase from late March (**Fig. 3D**). On the other hand, the transcript of *MdTFL1* decreased rapidly toward mid July before the peak of *MdFT1* expression in apical buds of FBS, with the signal in those of SS showing a delayed decrease at late September (**Fig. 3E**). For *MdAP1*, it started to increase from late August after the

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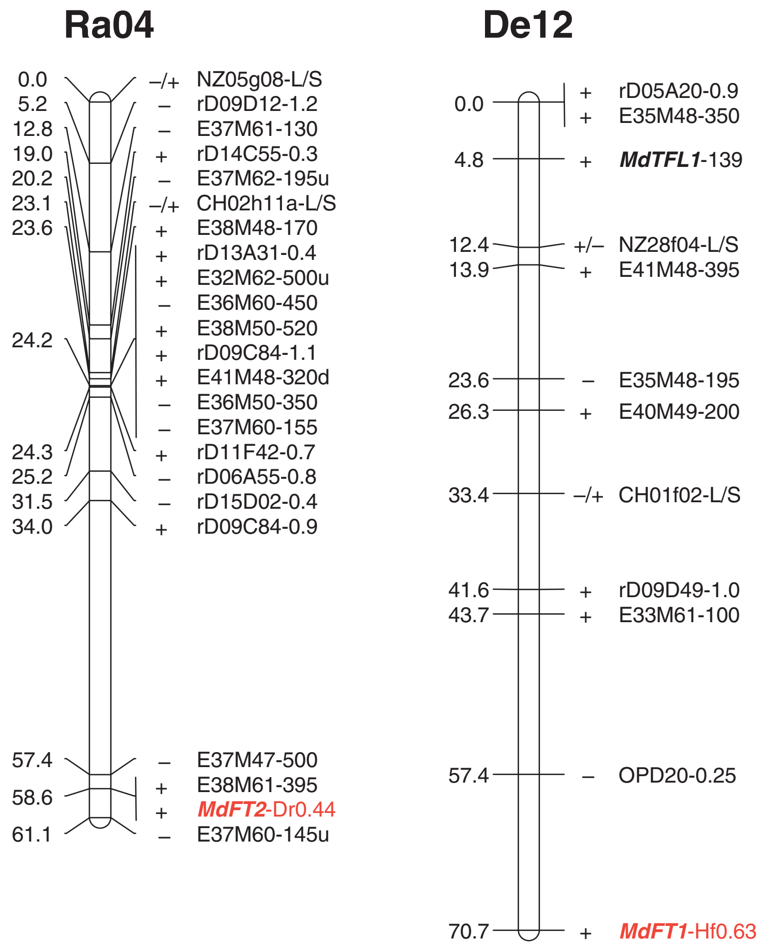

Fig. 2 Location of the *FT*-like genes on the linkage map of the apple cultivars 'Ralls Janet' (Ra) and 'Delicious' (De). *MdFT1* and *MdFT2* were located on LG 12 (De12) and LG 4 (Ra04), respectively. Linkage groups (LGs) of 'Ralls Janet' (Ra04) and 'Delicious' (De12) are derived from segregation data of 'Ralls Janet' and 'Delicious' populations, respectively. LG numbers are according to Maliepaard et al. (1998). Loci are listed on the right side of the linkage maps. The left side shows genetic distances (cM). The linkage phase information is provided as + or –, indicating on which of the homologous chromosomes the marker/allele is located. In the case of SSR markers, the sizes of SSR bands are shown by three-figure numbers or the relative sizes of the SSR bands are shown by the letter 'L (long)' or 'S (short)'. CAPS markers are shown with the gene name, the restriction enzyme (Dr, *Dra*I; Hf, *Hin*fI) and the length of the scored fragment (kbp). For a detailed explanation of molecular markers, see **Supplementary Table S2** of this paper and the materials and methods of Igarashi et al. (2008).

period of flower induction, consistent with the onset of the development of floral organ primordia (**Fig. 3F**).

Ectopic expressions of the apple *FT*-like genes in Arabidopsis

To determine the effects of apple *FT*-like genes on flowering time and inflorescence morphology, we generated two kinds of transgenic Arabidopsis plants with *MdFT1* or *MdFT2* under the control of the cauliflower mosaic virus (CaMV) 35S promoter fused with the Ω sequence (35SΩ) (**Fig. 4A**). Consequently, we obtained >20 independent transgenic lines for 35SΩ:*MdFT1* and 35SΩ:*MdFT2* (designated 35SΩ:*MdFT1*/wt and 35SΩ:*MdFT2*/wt, respectively). Among them, 10 independent lines per construct were used for further analysis. Eight to 10 plants in the T_2 generation per line were grown under long-day (LD, 16 h light/8 h dark) conditions, and their phenotypes were examined.

Seven of 10 transgenic Arabidopsis lines with 35SΩ:*MdFT1* flowered significantly earlier than wild-type plants under LD conditions at a *P*-value of <0.01 for the number of rosette leaves (**Fig. 4B**, **Table 1**). For example, 35SΩ:*MdFT1*/wt (#7) flowered with 3.9±0.2 rosette and 2.1±0.1 cauline leaves, while wild-type plants flowered with 6.7±0.3 rosette and 3.6±0.2 cauline leaves (**Table 1**). Nine out of 10 transgenic lines with 35SΩ:*MdFT2* also flowered significantly earlier than wild-type plants under LD conditions at a *P*-value of <0.01 for the number of rosette leaves (**Fig. 4B**, **Table 1**). For example, 35SΩ:*MdFT2*/wt (#3) exhibiting a strong phenotype flowered with 4.0±0.2 rosette and 1.9±0.2 cauline leaves, whereas wild-type plants flowered with 6.7±0.3 rosette and 3.6±0.2 cauline leaves (**Table 1**). No difference in the appearance of flowers and inflorescences was observed among 35SΩ:*MdFT1*/wt, 35SΩ:*MdFT2*/wt and wild-type plants (**Fig. 4C**). An early flowering phenotype was also observed in 35SΩ:*MdFT1*/wt and 35SΩ:*MdFT2*/wt under short-day conditions (data not shown).

Overexpression of *MdFT1* in apple

Transgenic experiments using Arabidopsis revealed that both *MdFT1* and *MdFT2* had the potential to function as floral promoters in apple. Expression analysis by qRT–PCR suggested that *MdFT1* played a role during floral transition, at least in tissues such as apical buds. Therefore, to clarify whether *MdFT1* confers an early-flowering phenotype in apple, we produced transgenic apples carrying the 35SΩ:*MdFT1* already used for

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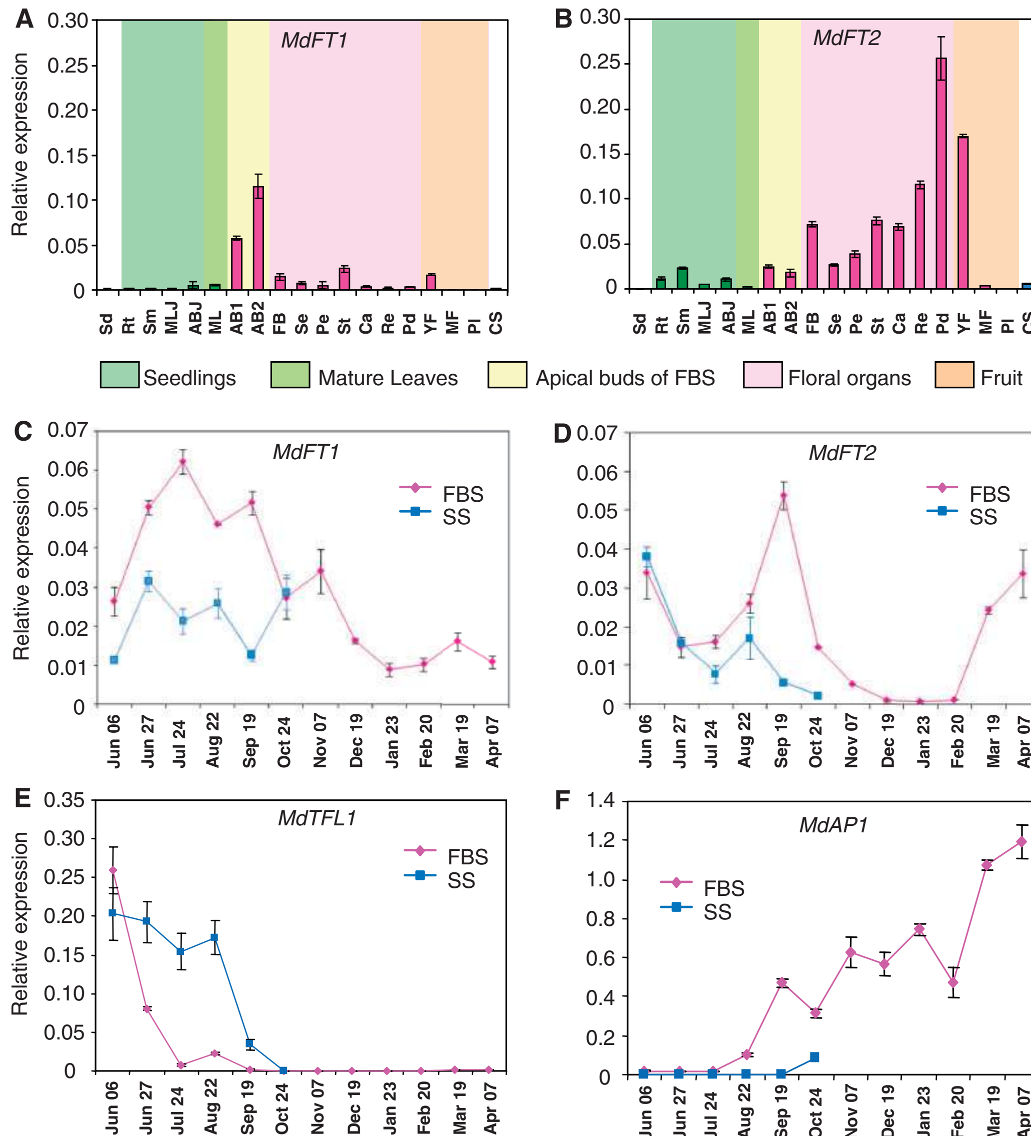

Fig. 3 Expression patterns of *MdFT1* (A) and *MdFT2* (B) in various tissues, and seasonal expression patterns of *MdFT1* (C), *MdFT2* (D), *MdTFL1* (E) and *MdAP1* (F) in apple by quantitative real-time RT–PCR. The samples for (A) and (B) from left to right are as follows: seeds (Sd), roots (Rt), stems (Sm), mature leaves (MLJ) and apical buds (ABJ) of 1-month-old seedlings (from a cross of cv. 'Fuji'×cv. 'Orin') in the juvenile phase; mature leaves (ML, collected on June 24) in the adult phase; apical buds of FBS on June 24 (AB1) and on August 7 (AB2) in the adult vegetative/reproductive phase; flower buds at the balloon stage (FB), sepals (Se), petals (Pe), stamens (St), carpels (Ca), receptacles (Re), peduncles (Pd), young fruit on June 15 (YF), mature fruit on November 18 (MF) and peel (Pl) from 'Fuji' apple in the adult reproductive phase; and shoots of tissue culture (CS). The samples for (C) to (F) from left to right are as follows: apical buds of fruit-bearing shoots (FBS) on June 6, June 27, July 24, August 22, September 19, October 24, November 7, December 19, January 23, Feburuary 20, March 19 and April 7; or succulent shoots (SS) on June 6, June 27, July 24, August 22, September 19 and October 24. Levels of detected amplicons were normalized by reference to amplified products that corresponded to apple *HistoneH3*. Values are means ± SD of results from three replicates. Each symbol without a bar indicates that the standard deviation fell within the symbol. The primer sets used in quantitative real-time RT–PCR analysis and the PCR conditions are described in **Supplementary Table S1**.

Arabidopsis transformation, using micropropagated tissues of an apple rootstock cv. 'JM2'.

As a result, we obtained six independent transgenic lines designated MdFT1#1-1, MdFT1#1-2, MdFT1#3, MdFT1#5, MdFT1#6, and MdFT1#8. Five of six transgenic lines (MdFT1#1-1, MdFT1#1-2, MdFT1#3, MdFT1#6 and MdFT1#8) produced flower buds or opened flowers *in vitro* 8–12 months after *Agrobacterium* infection (2–6 months after regeneration). For example, MdFT1#3 produced terminal flower buds at the top of each shoot, and some of them came into flower

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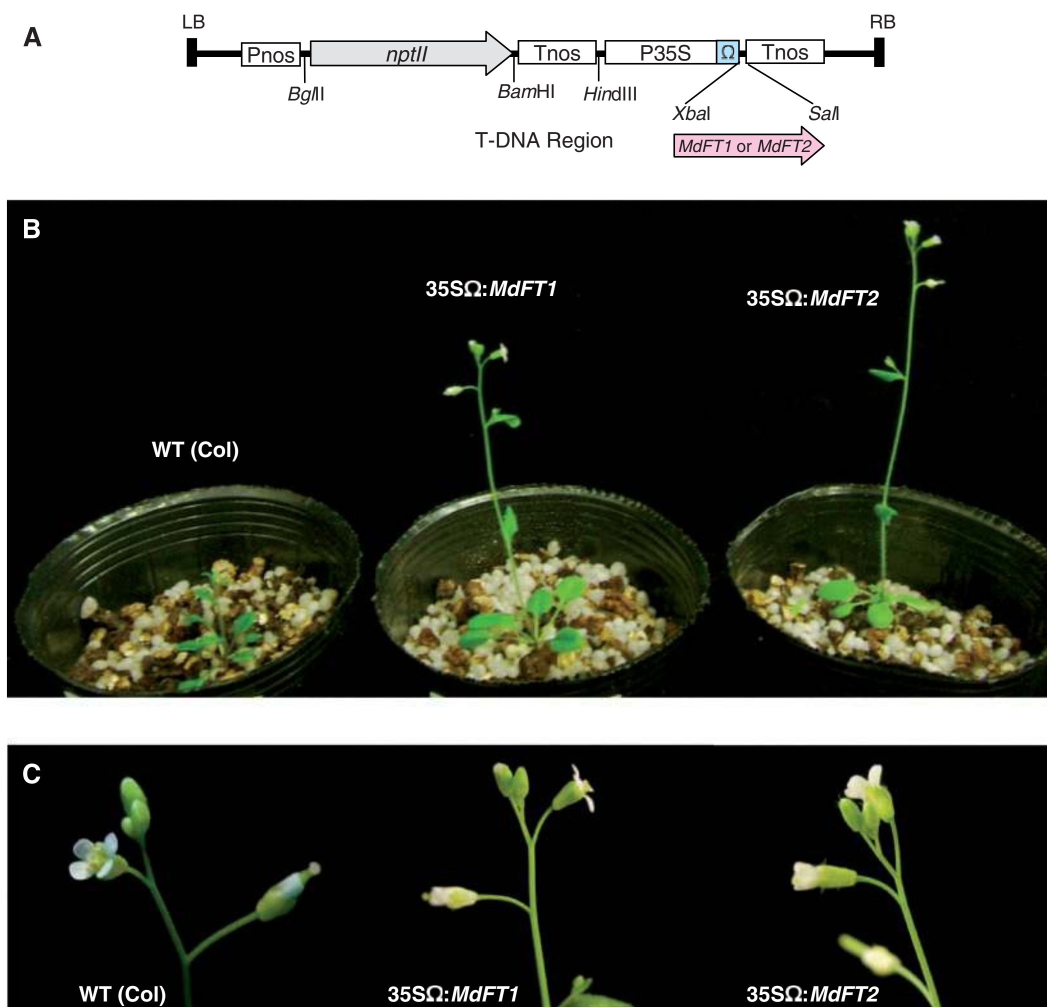

Fig. 4 Phenotypes of transgenic Arabidopsis with apple *FT*-like genes under long-day conditions. (A) Schematic representation of the transformation vectors, 35SΩ:*MdFT1* and 35SΩ:*MdFT2*. LB, left border; *nptII*, neomycin phosphotransferase II gene; P35S, promoter region of cauliflower mosaic virus 35S; Pnos, *nos* promoter; RB, right border; Tnos, 3′ region of *nos*. (B) Appearance of wild-type Arabidopsis (ecotype Columbia, left), transgenic Arabidopsis with 35SΩ:*MdFT1* (middle) and transgenic Arabidopsis with 35SΩ:*MdFT2* (right) 26 d after transfer to the growth chamber. (C) Inflorescence and flower of wild-type Arabidopsis (left), transgenic Arabidopsis with 35SΩ:*MdFT1* (middle) and transgenic Arabidopsis with 35SΩ:*MdFT2* (right). The photographs were taken after flowering for each plant. For details on the flowering time of transgenic lines, see **Table 1**. The wild type (Col) was used for Arabidopsis transformation. The plants in (B) and (C) were grown under LD conditions (16 h light/8 h dark) at 22°C.

(**Fig. 5A, B**), whereas the control plant never produced flower buds under the same culture conditions (**Fig. 5C**). In addition, the shoots of MdFT1#1-1 were grafted onto the apple rootstock ‘JM7’ and transferred to the greenhouse (**Fig. 5D**). The potted MdFT1#1-1 also produced a flower bud and opened a solitary flower; however, the flower had >8 petals (**Fig. 5E**), in contrast to the normal flower, which had five sepals, petals, carpels and approximately 20 stamens (**Fig. 5F**). Importantly, the *in vitro* flowering lines, such as MdFT1#3 and MdFT1#8, which produced *in vitro* flower buds more frequently than the others, showed a much less vigorous growth of shoots. Several lines with such severe phenotypes senesced, probably due to their inability to form new vegetative shoot meristems.

The result of DNA blot analysis for transgenic lines MdFT1#1-1, MdFT1#5 and MdFT1#8 indicated that MdFT#1-1 had two copies and the other lines had one copy of the transgene (**Fig. 5G**).

Influence of overexpression of *MdFT1* on the other apple genes

We examined the influence of the overexpression of *MdFT1* on the expression of other endogenous genes, which had been shown or expected to play a role in flowering in apple. To avoid the fluctuation of the gene expression by artificial and other environmental factors, we used the cultured shoots of each line grown in a growth chamber under the same conditions.

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Table 1 Flowering time of transgenic lines ectopically expressing *MdFT1* or *MdFT2*

Line	LD conditions (16 h light/8 h dark)			
	No. of plants	Rosette leaves	Cauline leaves	Total leaves
Wt (Col)	10	6.7±0.3	3.6±0.2	10.3±0.4
Vector/Wt (Col)	8	7.3±0.3	3.3±0.2	10.5±0.2
35SΩ:*MdFT1*/wt				
#1	9	4.0±0.3**	2.6±0.2**	6.6±0.4**
#2	10	3.9±0.2**	2.6±0.2**	6.5±0.2**
#3	10	6.2±0.2	3.1±0.1*	9.3±0.2*
#4	10	4.0±0.2**	2.2±0.1**	6.2±0.2**
#5	10	4.3±0.2**	2.1±0.1**	6.4±0.2**
#6	10	6.7±0.3	2.6±0.2**	9.3±0.4
#7	10	3.9±0.2**	2.1±0.1**	6.0±0.2**
#8	9	7.9±0.5	2.3±0.2**	10.2±0.4
#9	10	4.0±0.2**	2.8±0.2**	6.8±0.3**
#10	10	4.3±0.3**	2.3±0.1**	6.6±0.4**
35SΩ:*MdFT2*/wt				
#1	9	4.9±0.3**	2.1±0.1**	7.0±0.3**
#2	10	5.0±0.3**	3.0±0.2*	8.0±0.2**
#3	10	4.0±0.2**	1.9±0.2**	5.9±0.3**
#4	10	4.5±0.3**	2.3±0.1**	6.8±0.3**
#5	10	4.3±0.2**	2.2±0.1**	6.5±0.2**
#6	10	4.1±0.2**	3.3±0.2	7.4±0.4**
#7	9	5.3±0.3*	2.3±0.2**	7.7±0.4**
#8	8	4.4±0.4**	2.8±0.3*	7.1±0.3**
#9	10	4.9±0.1**	2.1±0.1**	7.0±0.0**
#10	10	4.3±0.1**	2.0±0.0**	6.3±0.1**

Plants in the second generation (T_2) were grown under long-day (LD) conditions. Numbers of rosette and cauline leaves were counted on the day floral organs became visible. Values are means±SE. Student's *t*-test was performed to compare the effects of treatments. One or two asterisks indicate a statistically significant difference from the wild-type plants in the same column (P <0.05, 0.01, respectively).

The result of qRT–PCR analysis showed that *MdMADS12* [a *FRUITFULL* (*FUL*)-like gene of apple; van der Linden et al. 2002] was significantly upregulated in the *in vitro* flowering lines MdFT1#1-1 and MdFT1#8, where the *MdFT1* transcripts accumulated in large numbers, whereas the expression level of *MdMADS12* in a line not flowering *in vitro*, MdFT1#5, was almost the same as that in the control (**Fig. 6A, B**). In addition, *MdSOC1a* and *MdSOC1b* (putative apple orthologs of *SOC1*; accession Nos. AB501124 and AB501125, respectively), and *MdAP1* (equivalent to *MdMADS5*, an apple ortholog of *AP1*) were more up-regulated in the lines MdFT1#1-1 and MdFT1#8, than in line MdFT1#5 and the non-transgenic control (**Fig. 6C–E**). The expression of *MdLHP1a*/*MdLHP1b* (*MdLHP1a* and *MdLHP1b*, apple homologs of *LHP1*/*TFL2*; Mimida et al. 2007) was not affected by the overexpression of *MdFT1* (**Fig. 6F**).

Discussion

MdFT1 and *MdFT2* are located in distinct linkage groups with duplicated segments

MdFT1 and *MdFT2* were mapped, respectively, on loci in distinct linkage groups, LG 12 and LG 4 (**Fig. 3**). Because there is a homoeology between the distal parts of LG 12 and LG 4 (Gardiner et al. 2007), *MdFT1* and *MdFT2* might have been derived from an ancient gene that ancestors of the original hybrid would have had. Coincidentally, *MdFT1* was located on the distal part of LG 12, the same linkage group as *MdTFL1*. This finding suggests that *MdFT1* and *MdTFL1* have evolved in close relation to each other and that at least one chromosome, corresponding to LG 12, plays an important role in the regulation of flowering in apple. To date, it has been revealed that apple

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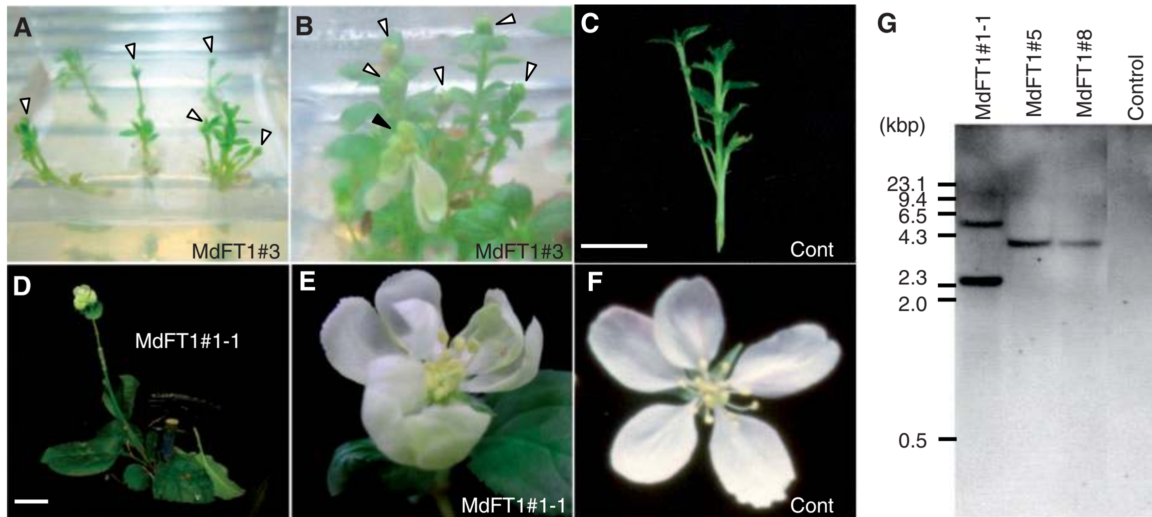

Fig. 5 Precocious flowering of transgenic apples overexpressing *MdFT1*. (A) Floral buds (indicated as an open triangle) formed at the top of the shoots of transgenic apple 'JM2' (line MdFT1#3) in the culture box. (B) *In vitro* flowering of a solitary flower (indicated as a filled triangle) at the top of the transgenic shoots in the culture box. (C) Cultured shoots of an apple 'JM2' control plant. A white bar indicates 1 cm. (D) Opened flower of transgenic apple 'JM2' (line MdFT1#1-1) in the greenhouse. A white bar indicates 3 cm. (E) Close-up view of (D). The number of petals increased compared with a normal flower with five petals. (F) Flower of an apple cv. 'JM2' control plant grown in the field. (G) DNA blot analysis of transgenic apples 'JM2' with 35SΩ:*MdFT1* (MdFT1#1-1, MdFT1#5 and MdFT1#8). Genomic DNA (15 µg) was isolated from the cultured tissues of each transgenic line and a control plant, digested with *Hin*dIII, and then separated on a 0.8% (w/v) agarose gel. A blotted membrane was hybridized with a DIG-labeled *nptII* gene.

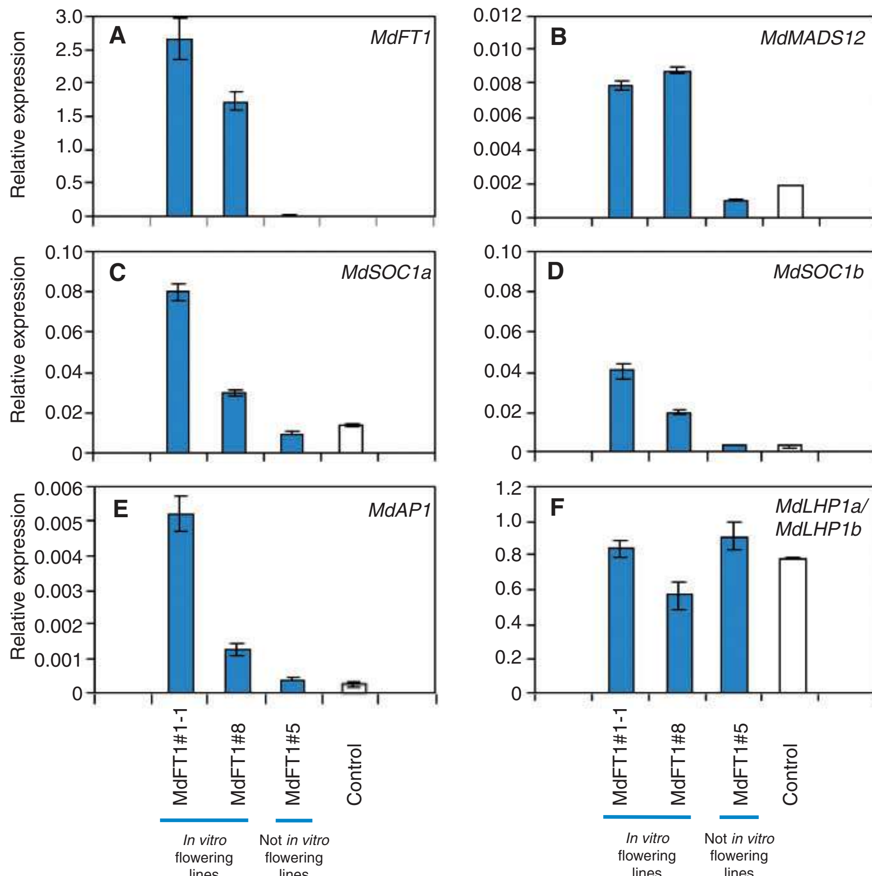

Fig. 6 Expression analysis for apple genes in the shoots of transgenic lines. (A–F) Expression of *MdFT1* (A), *MdMADS12* (B), *MdSOC1a* (C), *MdSOC1b* (D), *MdAP1* (E) and *MdLHP1a*/*MdLHP1b* (F) in the transgenic shoots of *in vitro* flowering lines (MdFT1#1-1 and MdFT1#8) and a line not flowering *in vitro* (MdFT1#5), and in non-transgenic shoots as a control. Shoots were collected from each line cultured in a proliferating medium under LD conditions for 4 weeks to synchronize the growth state of each line. *MdMADS12*, *MdSOC1a*, *MdSOC1b* and *MdAP1* are genes encoding a *MADS*-box transcription factor from apple; and *MdLHP1a*/*MdLHP1b* (*MdLHP1a* and *MdLHP1b*) are apple twin homologs of *LHP1*. Primers for *MdLHP1a*/*MdLHP1b* were designed to detect both *MdLHP1a* and *MdLHP1b*. *HistoneH3* was used as an internal control of gene expression. In A–F, non-transgenic apple cv. 'JM2' was used as a control plant (control). Transcript levels were normalized against apple *HistoneH3*. Values are means ± SD of results from three replicates, and each box without a bar indicates that the standard deviation fell within the box. The primer sets used in quantitative real-time RT–PCR analysis and the PCR conditions are described in **Supplementary Table S1**.

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has at least six members of the *TFL1*/*FT* family in its genome (Kotoda et al. 2005, Mimida et al. 2009, this study), although members that belong to the *BFT* or *MFT* clade have not been reported. Other eudicots, such as Arabidopsis and tomato, have about six *TFL1*/*FT* family genes (Mimida et al. 2001, Carmel-Goren et al. 2003). However, the Lombardy poplar genome has nine (Igasaki et al. 2008), probably due to a recent gene duplication event 8–13 million years ago (Tuskan et al. 2006). Because apple has duplicated chromosomal regions as well, further study might reveal additional members falling into the clade of *MFT* or *BFT* in the *TFL1*/*FT* family in apple.

MdFT1 and *MdFT2* show different expression profiles in apple

The expression pattern of *MdFT1* and *MdFT2* differed in that *MdFT1* was expressed mainly in apical buds of FBS in the adult phase, whereas *MdFT2* was expressed mainly in reproductive organs, including flower buds and young fruit (**Fig. 3A, B**). In addition, it was found that the transcripts of *MdFT1* could hardly be detected in the tissues of 1-month-old seedlings in the juvenile phase, in contrast to the transcripts of *MdFT2*. In Satsuma mandarin, two citrus homologs of *FT*, *CiFT1* and *CiFT2*, which are highly and specifically expressed in young fruit, were not involved in the transition to flowering, and only the third citrus homolog of *FT*, *CiFT3*, expressed at a relatively low level in leaves and stems but with no expression in reproductive organs, turned out to play a key role in regulating flower induction (Nishikawa et al. 2007). Considering that *MdFT2* was highly expressed in the reproductive organs and that it was expressed similarly in the apical buds of both FBS and SS during the period of flower induction, *MdFT1* might play a key role in the transition to flowering in apple (**Fig. 3**), although Arabidopsis *FT* and its paralog *TSF* are both highly expressed in flowers and developing siliques as well as in leaves (Kobayashi et al. 1999, Takada and Goto, 2003, Yamaguchi et al. 2005). The expression pattern of *MdFT1* in various tissues was somewhat similar to that of tomato *SFT* (formerly *SPD3*, a tomato ortholog of *FT*; Carmel-Goren et al. 2003, Lifshitz et al. 2006) rather than that of Arabidopsis.

In apple, flower induction occurs in late June [about 40–50 days after flowering (DAF)] and flower initiation with morphological changes at the apical meristem starts to form floral primordia in mid July (about 60 DAF) in Morioka, Japan (Kotoda et al. 2000). *MdFT1* was expressed to relatively higher levels in the apical buds of FBS than in those of SS from the period of flower induction to the early stage of flower development. This result was consistent with the observation that the expression of *MdTFL1* decreased rapidly from early June, although it is unclear where in apical buds *MdFT1* was expressed (**Fig. 3C, E**). Recently, Hättasch et al. (2008) reported that the increase in transcription of *MdFT* (*MdFT1* in this study), *AFL1* and *AFL2* (apple orthologs of *LFY*; Wada et al. 2002) in the apical meristems of current-year shoots of apple began 1–2 weeks earlier than flower initiation, suggesting that flower induction is initiated by these genes. The higher expression level of *MdFT2* in mid September and early April might be related to the development of floral organs in apple (**Fig. 3D**), because the expression pattern of *MdAP1* reflected the development of floral organs, such as sepals and receptacles (**Fig. 3F**, Kotoda et al. 2000).

MdFT1 and *MdFT2* have the potential to act as floral promoters and overexpression of *MdFT1* confers precocious flowering in apple

Transgenic Arabidopsis overexpressing *MdFT1* and *MdFT2* showed early flowering as compared with the control plant (**Fig. 4**, **Table 1**). Similar early-flowering phenotypes of transgenic Arabidopsis had been reported upon the ectopic expression of *FT*-like genes from heterologous plant species, such as citrus (Kobayashi et al. 1999), tomato (Teper-Bamnolker and Samach 2005, Lifschitz et al. 2006), grapevine (Sreekantan and Thomas 2006, Carmona et al. 2007), poplar (Hsu et al. 2006, Igasaki et al. 2008) and cucurbits (Lin et al. 2007), as well as the overexpression of *FT* and *TSF*. No differences in the extent of flower-promoting activity between *MdFT1* and *MdFT2* in transgenic Arabidopsis suggested that *MdFT1* and *MdFT2* both have the potential to act as floral promoters in apple (**Table 1**). However, expression analysis by qRT–PCR implied that, during floral transition, *MdFT1* plays a more important role, at least in tissues within the apical buds of FBS, where transition to flowering occurs.

Consequently, five out of six transgenic apples overexpressing *MdFT1* flowered *in vitro* 8–12 months after *Agrobacterium* infection (**Fig. 5**). As a whole, the precocity of flowering in *MdFT1*-overexpressing lines was much higher than that in previously reported *MdTFL1* down-regulated apples (Kotoda et al. 2003, Kotoda et al. 2006). The transgenic lines with 35SΩ:*MdFT1* that produced flower buds *in vitro* had a tendency to have small, rounded leaves and show an extremely weak growth habit. In the transgenic lines MdFT1#1-1 and MdFT1#8, which showed *in vitro* flowering, *MdFT1* was abundantly expressed, while the expression of *MdFT1* was extremely low in line MdFT1#5, possibly due to co-suppression (**Fig. 6A**). As expected, line MdFT#5 has not flowered *in vitro* or in pots for at least 3 years after regeneration. These results suggest that there is a positive correlation between *MdFT1* expression and flower induction in transgenic plants. Besides *MdFT1*, the heterologous gene *PnFT3* (a poplar ortholog of *FT*; Igasaki et al. 2008) also promoted the transition to flowering when overexpressed in the apple cultivar 'Greensleeves' (**Supplementary Fig. S2**). Apple trees normally produce five flowers per cluster, and each flower has five sepals, petals and carpels and approximately 20 stamens. The changes in the number of floral organs in the transgenic plant might result from disturbance of the expression of floral organ identity genes, such as *MADS*-box genes, due to the overexpression of *MdFT1* (**Fig. 5E**). Contrary to our results, Hättasch et al. (2009) reported that transgenic apples with *MdFT* did not flower for at least a year *in vitro* and in the greenhouse. This observation might be due to the fact that the regeneration competence of the transformed calli from some

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scion cultivars was severely impaired when *FT*-like genes were overexpressed, resulting in the selection of transgenic lines with little expression of those genes.

Up-regulation of MADS-box genes could result in *in vitro* flowering in the transgenic lines overexpressing *MdFT1*

QRT–PCR analysis revealed that *MdMADS12* and *MdAP1* were significantly more up-regulated in the cultured shoots of transgenic lines that highly expressed *MdFT1* than in those of line MdFT1#5, which had little expression of *MdFT1*, and a control (**Fig. 6**). These results seem to be consistent with the finding by Teper-Bamnolker and Samach (2005) that *FUL* and *AP1* were highly misexpressed in the young seedlings and the older rosette leaves of Arabidopsis overexpressing *FT*. Interestingly, Flachowsky et al. (2007) reported that the overexpression of *BpMADS4*, a birch (*Betula pendula*) *FUL*-like *MADS*-box gene (Elo et al. 2001), induced early flowering in the apple cv. 'Pinova', with frequent *in vitro* flowering. In addition, the phenotype of the transgenic apple with 35S:*BpMADS4* resembled that with 35SΩ:*MdFT1* in that it produced solitary flower buds and rounded leaves in tissue culture. Considering that *MdMADS12* is a putative ortholog of *FUL*, *MdMADS12* might play an important role in flower induction of apple downstream of *MdFT1*. In Arabidopsis, *FUL* is required in several developmental processes, including silique and leaf development, and in the transition to flowering (Gu et al. 1998, Ferrandiz et al. 2000, Teper-Bamnolker and Samach, 2005). On the other hand, the up-regulation of *MdAP1* in the transgenic lines indicated that the shoots had been florally induced because the expression of *MdAP1* starts to increase in the apical buds of FBS after flower initiation (**Figs. 3F** and **6E**; Kotoda et al. 2000). In addition, *MdSOC1a* and *MdSOC1b* were also up-regulated in the *in vitro* flowering lines with a similar expression pattern to *MdAP1* (**Fig. 6C–E**), implicating them as the common targets of *MdFT1*, as *SOC1* and *AP1* are activated by the FT–FD complex in Arabidopsis (Abe et al. 2005, Wigge et al. 2005, Michaels 2009).

Our results suggest that *MdFT1* could function upstream of those genes to regulate flowering in apple, although whether or not the regulation is direct remains to be demonstrated. In future studies, the interaction of partner genes, such as transcription factors, with *MdFT1* and/or *MdFT2* will be investigated.

Materials and Methods

Plant materials

The tissue samples of an apple (*Malus*×*domestica* Borkh.) cvs. 'Fuji' and 'Jonathan' in adult phase (age: 19–20 years) were collected from the experimental field at the National Institute of Fruit Tree Science in Morioka, Japan. One-month-old juvenile seedlings from a cross between apple cvs. 'Fuji' and 'Orin' were used for the expression analysis of apple *FT*-like genes in the juvenile phase. Micropropagated tissues of an apple rootstock cv. 'JM2' [Marubakaido 'Seishi' (*Malus prunifolia* Borkh. var. *ringo* Asami)×'Malling 9' ('M. 9')] (Soejima et al. 1998) and an apple cv. 'Greensleeves' were kept at 24°C under LD conditions (16 h photoperiod; cool white fluorescent light, 50 µmol m^{-2} s^{-1}) in the proliferating medium (MS medium containing B5 vitamin, 1 mg l^{-1} 6-benzyl-aminopurine, 0.1 mg l^{-1} indole-3-butyric acid) in the culture box and subcultured every 4 weeks. The leaf explants of those apple cultivars were used for *Agrobacterium*-mediated transformation. For Arabidopsis transformation, wild-type plants of ecotype Columbia (Col) were used.

Nucleic acid extraction and hybridization analysis

The genomic DNA was isolated by a cetyltrimethylammonium bromide (CTAB)-based method modified by Yamamoto and Mukai as described in Kotoda et al. (2002). The genomic DNA (15 µg) was digested with *Eco*RI, *Hin*dIII or *Xba*I, electrophoresed on a 0.8% agarose gel, and then blotted onto Hybond-N+ nylon membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The blotted membrane was hybridized with digoxigenin- (DIG; Roche Diagnostics, Mannheim, Germany) labeled *MdFT1* cDNA, which was amplified by PCR with a pair of primers, MdFT1(1→24) and MdFT1(508←528), and a DIG mixture (Roche Diagnostics). For DNA blot analysis of transformants, each genomic DNA of transgenic lines and a control plant was digested with *Hin*dIII and the blotted membrane was hybridized with a DIG-labeled *nptII* probe. The hybridization and washing was performed as described in Kotoda et al. (2002). Chemiluminescent signals were visualized using the LAS1000 image analyzer (Fuji Photo Film, Tokyo, Japan). The primer set is listed in **Supplementary Table S1**.

Isolation of *FT*-like genes from apple

The cDNA library derived from flower buds of 'Fuji' apple was constructed as described by Mimida et al. (2007). *MdFT1* (accession No. AB161112; Kotoda and Wada 2005) labeled with [α-^{32}P]dCTP (GE Healthcare Bio-Sciences Corp.) was used as a probe to screen 2.0×10^5 plaque-forming units (pfu) of the cDNA library. Positive plaques were excised to the pBluescript SK(–) phagemid (Stratagene, La Jolla, CA, USA), and approximately 20 clones were then sequenced using a DTCS Quick Start Kit for Dye Terminator Cycle Sequencing (Beckman Coulter, Fullerton, CA, USA) and an automated DNA sequencer CEQ 8000 (Beckman Coulter).

To obtain genomic DNA of *MdFT1*, the cDNA of *MdFT1* labeled with DIG was used as a probe to screen 2.0×10^5 pfu of the genomic library of 'Fuji' apple. The lambda clone DNAs from positive plaques were digested with restriction enzyme *Not*I and ligated into the corresponding site of pBluescriptII SK (+) (Stratagene). To obtain a *MdFT2* genomic sequence from apple, on the other hand, PCR amplification of DNA fragments was performed in a mixture of a pair of primers MdFT2-EcoRI(–171→–148) and MdFT2(842←859)-XhoI, a high-fidelity DNA polymerase (KOD plus; Toyobo, Osaka, Japan) and 250–300 ng of genomic DNA of 'Fuji' apple. PCR was programmed for pre-heating at 94°C for 2 min followed by

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30 cycles of 94°C for 30 s, 50°C for 30 s and 68°C for 2 min. The PCR-amplified fragments digested with *Eco*RI and *Xho*I were cloned into the corresponding site of pBluescriptII SK (+) (Stratagene), and then four clones were sequenced. The primer sets used in gene cloning are listed in **Supplementary Table S1**.

Mapping of FT-like genes on the linkage map of apple

Cleaved amplified polymorphic sequence (CAPS) markers for the *MdFT1* and *MdFT2* genes were developed, and genotyping was performed using an F_1 mapping population with 72 seedlings from the cross between 'Delicious' and Mitsubakaido (*Malus sieboldii* Rehder) and another population with 83 seedlings from the cross between 'Ralls Janet' and Mitsubakaido (Igarashi et al. 2008). A population from 'Delicious' and Mitsubakaido was used for the mapping of *MdFT1*. PCR was performed with a pair of primers MdFT1-5f and MdFT1-4r. A 632 bp product was digested with *Hin*fI, and the resultant 632 bp fragment specific to 'Delicious' was used for segregation analysis. On the other hand, a population from 'Ralls Janet' and Mitsubakaido was used for the mapping of *MdFT2*. PCR was performed with a pair of primers MdFT2-7f and MdFT2-8rm. A 1,064 bp product was digested with *Dra*I, giving a 435 bp fragment specific to 'Ralls Janet', which was used for segregation analysis. The linkage map was constructed using JoinMap ver. 3.0 (Van Ooijen and Voorrips 2001). Primer sets, restriction enzymes and PCR conditions for the CAPS markers are described in **Supplementary Table S2**.

Sequence analysis

Amino acid sequences were analyzed using the Clustal X multiple sequence alignment program ver. 1.83 (Jeanmougin et al. 1998) and BioEdit ver. 7.7.0 (H. Hall, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The phylogenetic tree was displayed using the N–J plot unrooted (Perrière and Gouy, 1996) with bootstrap values for 1,000 resamplings in each branch.

Expression analysis by q RT–PCR

For analysis, total RNA was extracted from flower buds (at the balloon stage), sepals, petals, stamens, carpels, receptacles, peduncles, young fruit, mature fruit, peel, mature leaves (collected on June 24) and apical buds of FBS (collected on June 24 and August 7) from adult 'Fuji' apple trees; leaves, apical buds of vegetative shoots, stems and roots from 1-month-old juvenile seedlings and seeds from a cross between 'Fuji' and 'Orin' apples; apical buds of FBS (from June to the following April in 2004) and SS (from June to October in 2003) in 'Jonathan' apple trees; *in vitro* cultured shoots of 'Fuji' apple; and transgenic and non-transgenic lines of 'JM2' apple by using a PolyA Tract mRNA purification kit (Promega, Madison, WI, USA). For seasonal expression analysis, apical buds of all samples from the orchard were collected between 10 and 14 h. The first-strand cDNAs were synthesized from 1 µg of total RNAs in 20 µl of a reaction mixture using a QuantiTect Reverse Transcription kit (Qiagen GmbH, Hilden, Germany). The subsequent PCRs were performed with 1 µl of the first-strand cDNA as templates in a total volume of 12.5 µl by using ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). Transcripts of *MdFT1*, *MdFT2*, *MdLHP1a/MdLHP1b* (*MdLHP1a* and *MdLHP1b*), *MdTFL1*, *MdMADS12*, *MdSOC1a*, *MdSOC1b*, *MdAP1* and *HistoneH3* were identified with the specific primers (the primer sets used in qRT–PCR analysis and the PCR conditions are described in **Supplementary Table S1**). An apple *HistoneH3* gene (Kotoda et al. 2006) was used as an internal control. QRT–PCR was performed three times and transcript levels were normalized against apple *HistoneH3*.

Construction of the transformation vector

To construct a vector for the constitutive expression of *MdFT1* and *MdFT2*, the coding region of each gene was amplified by PCR with thr pair of primers MdFT1-XbaI and MdFT1-SalI for *MdFT1*, and with thr pair of primers MdFT2-XbaI and MdFTa-SalI for *MdFT2* (**Supplementary Table S1**). An amplified PCR product was subsequently digested with *Xba*I and *Sal*I and then cloned into the *Xba*I/*Sal*I sites of the modified pBI221 (pBI9526Ω) to be placed between the CaMV 35S promoter fused with the Ω sequence (Gallie and Walbot 1992) and the terminator of the nopaline synthase (nos) (**Supplementary Fig. S1**). The resultant plasmid was cut with *Apa*I, and then the fragment containing *MdFT1* or *MdFT2* was ligated into the same restriction enzyme site of the pSMAK312Blue binary vector (pSMAK312Blue; H. Ichikawa, in preparation). For vector construction of 35SΩ::*PnFT3S*, poplar *PnFT3S* amplified by PCR was inserted into the modified pSMAK193E (pSMAK193E; H. Ichikawa, in preparation) to be placed between 35SΩ and the 3′ region of the Arabidopsis rbcS-2B gene (TrbcS).

Arabidopsis transformation

Agrobacterium tumefaciens strain EHA101 was used to transform *A. thaliana* (Col) plants by the floral-dip method (Clough and Bent 1998). Kanamycin-resistant transformants were transplanted from the plate to moistened potting soil composed of vermiculite and perlite [1 : 1 (v/v)] after 2–5 adult leaves had developed and grown in the growth chamber (Biotron, Nippon Medical and Chemical Instruments Co., Ltd., Tokyo, Japan) set at 22°C under LD conditions (16 h photoperiod; cool white fluorescent light, 50 µmol m^{-2} s^{-1}). Morphological analyses were performed on the second generation (T_2).

Apple transformation

The apple cvs. 'JM2' and 'Greensleeves' were transformed with *A. tumefaciens* strain EHA101 bearing 35SΩ:*MdFT1* and 35SΩ:*PnFT3* (*PnFT3*; a poplar ortholog of *FT*), respectively. *Agrobacterium tumefaciens* was cultured overnight on a shaker in 20 ml of liquid LB medium with 100 mg l^{-1} trobicin (Pfizer Japan, Tokyo, Japan) at 28°C. After centrifugation, the pellet was resuspended with MS medium (Murashige and Skoog 1962) and further diluted to an optical density (OD) of 0.5–0.8 at 600 nm. Leaf explants were infected with the inoculum for

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30 min. The selection of transgenic shoots for 'Greensleeves' was performed according to the procedure described by James et al. (1989) and Yao et al. (1995) and the procedure for 'JM2' will be published elsewhere (S. Takahashi, in preparation). Transformed shoots were multiplied in the proliferating medium supplemented with 50 mg l^{-1} kanamycin. Two to three shoots per line were grafted to apple rootstock 'JM7' [Marubakaido 'Seishi' (*Malus prunifolia* Borkh. var. *ringo* Asami) × 'Malling 9' ('M. 9')] (Soejima et al. 1998). Grafted apples were grown in an isolated greenhouse under natural day-length with the temperature set at 20–25°C during the growing season as described in Kotoda et al. (2006).

Supplementary data

Supplementary data are available at PCP online.

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