Chapter 3

Characterization of transgenic *Arabidopsis* expressing *MdAP1*

3.1 Abstract

An apple (*Malus×domestica* Borkh.) gene *MdMADS5*, a putative homolog of *Arabidopsis APETALA1* (*AP1*) and *Antirrhinum SQUAMOSA* (*SQUA*), was introduced into *Arabidopsis* using *Agrobacterium tumefaciens* EHA101 to examine how the gene affects flowering and whether it may be effective in producing transgenic apple that flowers early. Five of the 15 primarily transformed (T_0) plants flowered five to ten days earlier than wild-type plants and produced only two to three rosette leaves when they flowered. The terminal flowers on the transgenic plants resembled transgenic *Arabidopsis* expressing 35S::*AP1* or *tfl1* mutant plants. This phenotype observed in T_1 transgenic plants was inherited in the following generation. Based on these results, it was suggested that the function of the *MdMADS5* gene was similar to that of the *AP1* gene. Although the mechanism of flower-bud formation in the apple might be different from that in *Arabidopsis*, the *MdMADS5* gene might be involved in flower-bud formation of the apple.

3.2 Introduction

Arabidopsis thaliana has been widely used in studies on basic plant physiology and molecular biology because of its small genome, short regeneration time, self-compatibility, and ease in efficient regeneration and transformation procedures. The most striking advances in our

understanding of the genetic control of the timing of flowering have come from work on *Arabidopsis* (Levy and Dean, 1998; Pidokowich et al., 1999; Piñeiro and Coupland, 1998). Genes that control flowering time have been isolated. They include *LEAFY* (*LFY*) (Schultz and Haughn, 1991; Weigel et al., 1992), *APETALA1* (*AP1*) (Irish and Sussex, 1990; Mandel et al., 1992), *CAULIFLOWER* (*CAL*) (Kempin et al., 1995), and *TERMINAL FLOWER1* (*TFL1*) (Bradley et al., 1997; Ohshima et al., 1997; Shannon and Meeks-Wagner, 1991) in *Arabidopsis.* Over expression of *LFY* (Weigel and Nilsson, 1995) and *AP1* (Mandel and Yanofsky, 1995) shortened a juvenile period and caused early flowering, whereas overexpression of *TFL1* caused late flowering (Ratcliffe et al., 1998).

Genetic studies on morphogenesis in apple have been started since genes related to plant development including flower initiation were isolated in *Arabidopsis.* In apple, a MADS-box gene (Riechmann and Meyerowitz, 1997), *MdMADS1* (Sung and An, 1997), and *Knotted1*-like homeobox genes (Watillon *et al.,* 1997) were isolated first based on work in *Arabidopsis* and maize (*Zea mays* L.), respectively. Recently, Yao *et al.* (2001) have cloned the apple *PISTILLATA* (*PI*) (Goto and Meyerowitz, 1994) homologues (*MdPI*) and identified that in apetalous mutants such as 'Rae Ime' and 'Spencer Seedless', *MdPI* has been mutated by a retrotransposon insertion. As for genes homologous to *AP1*, *MdMADS2* (Sung et al., 1999) and *MdMADS5* (Yao et al., 1999) were cloned, and overexpression of *MdMAD2* caused early flowering in tobacco (*Nicotiana tabacum* L.). *MdMADS5* was expressed in skins and cortexes (Yao et al., 1999) and, two months after flower-bud differentiation, in sepals (Kotoda et al., 2000) in apple.

In this section, we describe the effect of an apple *MdMADS5* gene, a putative homologue of *AP1*, on flowering time in transgenic *Arabidopsis.*

3.3 Materials and methods

3.3.1 Plant materials

The apple (*Malus* x *domestica*) var. Jonathan and *Arabidopsis* ecotype Columbia (Col) were used in this study. Apple leaves and flowers were collected from the experimental field at our research center in Morioka, Japan.

3.3.2 Extraction of DNA from apple

DNA was isolated from the 'Jonathan' apple [age: 15-16 yr; rootstock: Maruba kaido (*Malus prunifolia* var. *ringo* Asami); location: Morioka, Japan] in 1998 by a cetyltrimethylammonium bromide (CTAB)-based method modified by Yamamoto and Mukai as described in the following. Approximately 3 g of young leaves of apple were frozen in liquid nitrogen and ground into fine powder. The powder was mixed with 20 mL of isolation buffer (IB) (10% polyethylene glycol 6000, 0.35 M Sorbitol, 0.1 M Tris-HCl pH 7.5, and 1% 2-mercaptoethanol) in a 50-ml centrifugation tube on ice for 2 min. The tube was rotated at 12,000 g_n for 5 min at 25 °C by swing rotor KS-5000P (Kubota Co., Tokyo, Japan). The pellet was then remixed with 9 mL of lysis buffer (LB) (0.35 M Sorbitol, 0.1 M Tris-HCl pH 7.5, and 1% 2-mercaptoethanol), and 1 mL of 10% sarcosine was added to it. The solution was mixed gently (about 50 rpm) at 25 °C for 10 min using a hybridization oven (Iwaki Glass Co., Ltd., Tokyo, Japan), and 10 ml of 2×CTAB (2% CTAB, 0.1 M Tris-HCl pH 9.5, 20 mM EDTA, 1.4 M NaCl, and 1% 2-mercaptoethanol) was added to it, followed by gentle mixing at 56 °C for 20 min. The solution was extracted twice with chloroform/isoamyl alcohol [24:1 (v/v)], and DNA in the aqueous phase was precipitated with an equal volume of 2-propanol at 25 °C. Nucleic acids were collected with an inoculation roop, then resolved in 5 mL of 1M NaCl containing RNase at a concentration of 10 mg · mL^{-1} in 15 mL tube, and incubated at 56 °C for 2-3 hr. The DNA was

precipitated with 10 mL of ethanol and washed twice with 2 mL of 70% ethanol for 5 min. After air drying, the pellet was dissolved in 1/10 TE (1 mM Tris-HCl pH 8.0, and 0.1 mM EDTA) and stored at 4 °C.

3.3.3   Extraction of RNA from *Arabidopsis*

RNA was isolated from *Arabidopsis* with a method using cetyltrimethylammonium bromide (CTAB) (Chang et al., 1993) that was modified by Yamamoto and Mukai as follows. Approximately 0.1 g of plant tissue was frozen in liquid nitrogen and then ground into fine powder. The powder was mixed with 1 mL 2×CTAB (2% CTAB, 0.1 M Tris-HCl pH 9.5, 20 mM EDTA, 1.4 M NaCl, and 1% 2-mercaptoethanol) and incubated at 65 °C for 10 min. The solution was extracted twice with chloroform/isoamyl alcohol [24:1 (v/v)], and RNA in the aqueous phase was precipitated with 0.25 volume of 10 M lithium chloride at -20 °C for 2 h. Nucleic acids were pelleted by centrifugation at 12,000 g_n for 10 min at 4 °C. The pellet was dissolved in TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA) and then extracted with TE-saturated phenol and phenol/chloroform. The aqueous layer was extracted sequentially with chloroform/isoamyl alcohol [24:1 (v/v)], and then RNA in the aqueous phase precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. RNA was pelleted by centrifugation at 12,000 g_n for 10 min at 4 °C. The pellet was dissolved in TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA).

3.3.4   DNA blotting analysis

DNA was obtained from young leaves of a 'Jonathan' apple. Ten μ g of the genomic DNA was digested with restriction enzymes, *Eco*RI, *Bam*HI, and *Hin*dIII according to manufacturer's instructions and electorophoresed on 0.8% Seakem GTG agarose gel (FMC Bioproducts, Rockland, Maine, USA). The gel was blotted onto Hybond N$^+$ (Amersham, Buckinghamshire, UK) and hybridized with a digoxigenin (DIG)-labeled PCR probe. A 567-bp fragment of

MdMADS5 cDNA was used as a template of PCR probes for DNA gel blot analysis. Hybidization was performed at 65℃ in a 0.5 M Na-Pi buffer (0.5 M Na_2HPO_4, pH 7.2), 7% SDS, and 1 mM EDTA for 16 hr followed by three washes in a 40 mM Na-Pi buffer (pH 7.2) and 1% SDS at 65 ℃ for 20 min. The following washes and detection were performed according to the protocol provided by the manufacturer.

3.3.5   Gene cloning of *MdMADS5*

Homologue fragments of *SQUA/AP1* were amplified by reverse transcription (RT)-polymerase chain reaction (PCR) from the sepals of mature floral buds of the 'Jonathan' apple. Two degenerate oligonucleotides (squa/ap1-1S sense primers and a squa/ap1-5A antisense primer) were used to amplify a homologous sequence of *SQUA/AP1* from apple cDNA for the RT-PCR (Table 3-1). 567-bp internal fragments were amplified and cloned into pBluescript II SK+ vectors (Stratagene, La Jolla, CA, USA). The cDNA library was constructed for cloning by rapid amplified cDNA ends (RACE)-PCR (Chenchic *et al.*, 1996) using a TaKaRa LA PCR™ *in vitro* cloning kit (Takara Biomedicals, Tokyo, Japan). After the cDNA synthesis from mRNA in sepals of apple, an *Eco*RI cassette was ligated to the cDNA. Then, 5' RACE and 3' RACE-PCR were performed using a set of adaptor primers and 2 antisense primers, and a set of adaptor primers and 2 sense primers, respectively   (Table 3-1). The corresponding 1.0-kb cDNA was amplified by using long and accurate (LA) -PCR (Barnes, 1994). A specific sense primer 5'-CAG TTT CTG GGT TGT CTT TC-3' derived from information of gene bank data registered by Yao et al. (1999) and a specific antisense primer 5'-ACT CAA TAT TTC TGA GGT TT-3' derived from a 600-bp fragment amplified by 3' RACE-PCR were used. The PCR products were cloned into a t-tailed *Sma*I site of pUC119 vectors (Takara Biomedical). The sequences of several clones of inserts in pUC119 were analyzed by dideoxy methods using a Thermo Sequenase pre-mixed cycle sequence kit according to the manufacturer's instructions (Amersham, Buckinghamshire, UK) and universal

primers in the pUC119 vectors on a Hitachi SQ5500 automated sequencer (Hitachi, Tokyo, Japan).

3.3.6 Vector construction for the expression of the *MdMADS5* gene

The pUC119 plasmid vector, pUMDAP1.2+, containing *MdMADS5* cDNA, was digested with *Xba*I and *Sac*I, ligated to the binary vector pSMAK 251(Yamashita et al., 1995) [similarly digested with *Xba*I and *Sac*I to remove the β-glucuronidase (GUS) gene] in a sense-oriented manner, resulting in the binary vector pSMDAP1.1+. The binary vector pSMDAP1.1+ was then introduced into a disarmed strain of *Agrobacteriuim tumefaciens*, EHA101 (Hood et al., 1986).

3.3.7 *Arabidopsis* transformation

An *Agrobacteriuim tumefaciens* strain EHA101 was used to transform *Arabidopsis* Columbia plants (Col) by a floral-dip method (Clough and Bent, 1998). *Agrobacteriuim tumefaciens* were grown at 27°C in sterilized ϕB medium (20 g $\cdot$ L^{-1} Bacto tryptone, 5g $\cdot$ L^{-1} Bacto yeast extract and 10 g$\cdot$L^{-1} MgSO$_4$) carrying added 25 μl$\cdot$mL^{-1} trobicin (spectinomycin) and 25 μl$\cdot$mL^{-1} streptomycin. Cells were harvested by centrifugation for 5 min at room temperature at 4,800 g_n. The inoculum was resuspended to OD$_{600}$ = 0.8 in 5% sucrose and 0.005% surfactant Silwet L-77 (Nippon Unicar Co., Ltd., Tokyo, Japan). Plants were inoculated by dipping developing floral tissues when numerous immature floral buds emerged. Plants grew for 3-5 weeks more until the siliques were brown and dry. Seeds were harvested by hand and stored at 4 °C for at least two days. Seeds were treated with 70% ethanol for 1 min and then with 2.5% sodium hypochloride containing 0.05% Tween 20 for 5 min, followed by three rinses with sterile water. To select transformed plants, the sterilized seeds were suspended in 0.1% sterile agarose, plated on kanamycin selection plates, and transferred to a growth chamber BIOTRON (Nippon Medical and Chemical Instruments Co., Ltd., Tokyo, Japan) set at 22 °C under long day

conditions (16 hr light / 8 hr dark). The selection plates contained 1/2 Murashige and Skoog (MS) medium (Wako Pure Chemicals Co., Ltd., Tokyo, Japan), 0.8% agar (Difco Laboratories, Detroit, MI, USA), 25 μg · mL^{-1} kanamycin monosulfate (Meiji Seika Kaisha Ltd., Tokyo, Japan) and 50 μg · mL^{-1} claforan (Pharmacia and Upjohn Ltd., Tokyo, Japan). Transformants were identified as kanamycin-resistant seedlings that produced green leaves and well-established roots in the medium. Transformants were transplanted into moistened potting soil consisting of vermiculite and perlite [1:1 (v/v)] after they developed 2-5 adult leaves. The day of sowing was counted as day 0. Morphological analyses were performed in the primary (T_0) and subsequent (T_1, T_2) generations.

3.3.8 RNA analysis by RT-PCR

Detection of *MdMADS5* by RT-PCR was performed by using the RT-PCR high (Toyobo, Tokyo, Japan). As an internal control, the actin *AAc1* gene (Nairn *et al.*, 1988) was employed. Total RNA was isolated from the whole plant above the soil by a CTAB-based method. *MdMADS5-* and *AAc1* specific transcripts were identified using 1 μg of total RNA as a template and the following primers: a sense primer 1S: 5'-CAG TTT CTG GGT TGT CTT TC-3' and an antisense primer 7A: 5'-ACT CAA TAT TTC TCA GGT TT-3' (Table 3-1) for *MdMADS5*, and a sense primer Aac1-S: 5'-GTG CTC GAC TCT GGA GAT GGT GTG-3' and an antisense primer Aac1-AS: 5'-CGG CGA TTC CAG GGA ACA TTG TGG-3' for *Aac1*, giving rise to a 1037- and 457-bp long PCR product, respectively. PCR reactions were run for 40 cycles at 50 ℃ for *MdMADS5* and at 58 ℃ for *AAc1*. The PCR products were run on 1.5% (w/v) agarose gel stained with ethidium bromide.

3.3.9 Light microscopy

For magnification, the plants were viewed with an Olympus SZ-PT microscope (Olympus, Tokyo, Japan), and photographs were taken using Fujichrome Sensia II 36 reversal film (Fujifilm, Tokyo, Japan). Other photographs were taken using a digital camera Camedia C-2500L (Olympus, Tokyo, Japan).

3.4 Results

3.4.1 Gene cloning

A PCR technique was used to clone an *AP1*-like gene from apple. Two degenerated primers specific to the *AP1* and *SQUA* (Huijser et al., 1992; Schwarz-Sommer et al., 1990) genes were used to amplify fragments from cDNA synthesized from mRNA isolated from sepals of apple, and a 567-bp internal fragment was amplified by RT-PCR (Kotoda et al., 2000). Based on the internal fragment, the 1037-bp long cDNA of *MdMADS5* was obtained from the 'Jonathan' apple using gene bank data on *MdMADS5* first registered by Yao et al. (1999) and sequence data of the RACE-PCR (Chenchic et al., 1996) product. The deduced amino-acid sequence of *MdMADS5* in the coding region is 66% and 70% identical to *AP1* and *SQUA*, respectively (Fig. 3-1). DNA blotting was performed using apple DNA digested with *Bam*HI, *Eco*RI, and *Hin*dIII probed with a 567-bp long *MdMADS5* cDNA fragment. Two bands for *Bam*HI and *Eco*RI, and four bands for *Hin*dIII were detected in DNA blotting analysis, suggesting that the there might be a gene similar to *MdMADS5* (Fig. 3-2). In fact, *MdMADS2*, reported by Sung et al. (1999), was similar to *MdMADS5* with 62% amino-acid identity between them.

3.4.2 Construction of pSMDAP1.1+

The brief map of pSMDAP1.1+ is shown in Fig. 3-3. The pUC119 plasmid vector, pUMDAP1.2+, containing *MdMADS5* cDNA, was digested with *Xba*I and *Sac*I to give a fragment containing 1037-bp-long *MdMADS5* cDNA. The *MdMADS5* cDNA was inserted between the *Xba*I and *Sac*I sites of the binary vector pSMAK251 (Yamashita et al., 1995) downstream of the CaMV 35S promoter in sense orientation. The binary plasmid backbone was derived from the pKM304 (Murase, unpublished results). The sequences related to stabilization of the vector (sta) and replication (rep) are derived from *Pseudomonas* plasmid pVS1 (Itoh et al., 1984) and were introduced into pSMAK251. The *MdMADS5* is regulated by the CaMV 35S promoter and the *rbcS* terminator. The selectable marker gene encodes kanamycin resistance for plant and spectinomycin resistance for bacteria. The binary vector pSMDAP1.1+ was introduced into the *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986) for plant transformation, as described by Holsters et al. (1978).

3.4.3 Transgenic *Arabidopsis* expressing *35S::MdMADS5*

In order to examine whether *MdMADS5* functions like *AP1*, we produced transgenic *Arabidopsis* ecotype Columbia plants carrying the cDNA of *MdMADS5* fused to the CaMV 35S promoter. By floral-dip method, 15 independent transgenic plants that survived on kanamycin were identified (Table 3-2). Five of the 15 primarily transformed plants (T_0 generation) carrying *35S::MdMADS5* flowered earlier than the wild-type plants by five to ten days and produced only two to three rosette leaves when they flowered. The phenotype in lines 1 and 2 was so severe that it was difficult to obtain their seeds. The terminal flowers on the transgenic plants resembled those of *tfl1* mutant plants (Shannon and Meeks-Wagner, 1991). Similar results were obtained in T_0 transgenic *Arabidopsis* with *35S::AP1* (data not shown). This phenotype observed in T_0 transgenic plants was inherited in the following generation as a dominant Mendelian trait and co-segregated

with kanamycin resistance. All transgenic plants with early flowering contained a kanamycin resistance gene. T_1 Transgenic line No. 12-1 showed early flowering although no differences in appearance were seen between T_0 transgenic line No. 12 and the wild-type plants. Quantitative characteristics of five independent transgenic lines (T_2 generation) are shown in Table 3-3. Typical 35S::*MdMADS5* transformants (T_3 seedlings from the transgenic line No. 3-1-7) that flowered 17 days after sowing are shown in Fig. 3-4A and B. The wild-type plant had eight rosette leaves and no flower buds on day 20 (Fig. 3-4C). The inflorescence of transformants with early flowering was shorter (< 3 cm) and numbers of rosette leaves were extremely reduced (two to five) at flowering as compared to controls (Fig. 3-4B and Table 3-3). In wild-type *Arabidopsis*, flower consists of four floral organs (four sepals, four petals, six stamens, and a pistil) (Fig 3-4D, E). However, in transgenic lines with a severe phenotype, abnormal floral organs were observed (Fig. 3-4F, G) and some were less fertile.

3.4.4   Expression analysis by RT-PCR

The expression of the *MdMADS5* mRNA in T_0 plants was confirmed by RT-PCR (Fig. 3-5). The total RNA was extracted from whole plants of each transgenic line, and reverse transcription was done using random primers, followed by PCR using 1S and 7A primers (Table 3-1) specific to *MdMADS5*. All putative transformed lines showed expected RT-PCR products of 1037-bp for *MdMADS5*. No amplification was observed for the cDNA prepared from non-transformed *Arabidopsis* plants (Fig. 3-5, wt). The expression of *MdMADS5* mRNA was detected in all of the kanamycin-resistant plants except for the transgenic lines Nos. 7 and 8, which were not tested for expression analysis. The *MdMADS5* mRNA was expressed relatively weakly in five lines that flowered early (Fig. 3-5, lanes 1-5), compared to other lines. It was detected slightly in the line No. 5 plant, although the line No. 5 plant flowered earlier and produced far fewer rosettes than the wild-type plants.

3.4.5   The effect of day-length on the flowering of transgenic *Arabidopsis*

The time of flowering is regulated by endogenous signals and environmental conditions such as day-length. As *A. thaliana* is a quantitative long-day (LD) plant, LD photoperiods accelerate flowering, although plants also flower under short-day (SD) after extended periods of vegetative development. To study the effect of day-length on flowering in transgenic plants with 35S::*MdMADS5*, T_2 transgenic *Arabidopsis* plants were grown under both LD (16 hr light / 8 hr dark) and SD (8 hr light / 16hr dark) photoperiods (Table 3-3). Ten to 15 seeds from each T_1 transgenic line (Nos. 2-3, 3-1, 4-1, 5-3, and 12-1) derived from T_0 lines (Nos. 2, 3, 4, 5, and 12, respectively) were used in this study. With LD photoperiods, seedlings from lines Nos. 2-3, 3-1, and 12-1 showed severe phenotypes, and those from lines Nos. 4-1 and 5-3 showed milder ones. Most plants from several lines with a strong phenotype, such as line No. 2-3, died as a result of poor growth from the effects of the transgene, resulting in few seeds being obtained from them. In SD photoperiods, on the other hand, all transformants flowered earlier than the wild-type plants as they did in LD photoperiods. Seedlings from line No. 2-3 showed an especially severe phenotype of all transgenic lines with little difference in flowering time and the number of rosette leaves compared to the corresponding seedlings under LD photoperiods. In this transgenic line, flowering time and amount of vegetative development did not seem to depend on day-length. The other transgenic lines were delayed in flowering under SD photoperiods compared to those grown in LD photoperiods, but they flowered 4-6 weeks earlier than the wild-type plants that required about nine weeks to flower.

3.5 Discussion

We have produced transgenic *Arabidopsis* plants expressing *MdMADS5*. They flowered

earlier and had a shorter inflorescence and reduced number of rosette leaves compared to the controls (Fig. 3-4 and Table 3-3). The *MdMADS5* gene apparently caused early flowering in *Arabidopsis*, and our results showed that the *MdMADS5* gene might have a similar function to that of *AP1*. In our previous study, we showed that the *MdMADS5* gene was expressed in sepals and that it was first expressed two to three months after floral-bud differentiation concurrent with sepal formation of apple (Kotoda et al., 2000). Therefore, it was suggested that the *MdMADS5* gene is involved in flower development after floral-bud differentiation, although the mechanism of flower-bud formation in apple might be different from that in *Arabidopsis*. To be more certain that the *MdMADS5* gene functions similarly to *AP1*, *ap1* mutant-rescue experiments will be required. Based on the result that transgenic *Arabidopsis* with 35S::*MdMADS* flowered earlier than the controls under both LD and SD photoperiods, the *MdMADS5* gene does not seem to have an effect on a photoperiodic pathway in *Arabidopsis*. The *MdMADS5* gene was not expressed strongly in transformants showing early flowering relative to those that were wild type in appearance. The result suggests that the severity of phenotype in transformants does not depend solely on the level of gene expression in this case, although an exact answer to the cause was not obtained.

The breeding of fruit trees such as apple (*Malus × domestica*) often requires more than 20 years, including periods of cross pollination, seedling selection, and regional trials, to produce varieties that meet the demands of consumers. For example, the 'Fuji' apple, a leading cultivar in Japan, Korea, and China, took 23 years before it was released (Sadamori et al., 1963). Thus, one of factors that limits breeding strategies in tree fruits is the long juvenile phase that lasts several years (Hackett, 1985). Several years ago, it was found that transgenic approaches could reduce the juvenile phase of *Populus* by introducing *LFY* under the CaMV 35S promoter and the transgenic poplar flowered five months after regeneration (Weigel and Nilsson, 1995). Therefore, these techniques could be applicable to fruit trees in future years. We believe that the transgenic

approach would be a useful breeding strategy for reducing the time required for generation among woody plants. In addition to this study on *MdMADS5*, we confirmed that both *AFL1* and *AFL2*, twin apple homologs of *LFY*, also cause early flowering in *Arabidopsis* (Wada et al., 2002). Recently, Pena et al. (2001) reported that citrus expressing *LFY* or *AP1* were early flowering and they were fertile. However, the efficiency of producing early flowering transgenic lines with *Arabidopsis LFY* or *AP1* genes seems to be low in woody plants mentioned above. This suggests that regulatory genes such as *LFY* and *AP1* do not always function beyond species as well as expected. Based on these results, we are producing transgenic apples expressing endogenous apple genes, *AFL* or *MdMADS5*, for early flowering. Since environmental and genetic factors controlling flower development in apple have not been made clear so far, the *MdMADS5* gene could, at least, be one of the tools available for studying the mechanism of flower development in tree fruits such as the apple.

Table 3-1. Primers used for PCR cloning of *MdMADS5*

Primer	Oligonucleotide
Degenerate primers	
squa/ap1-1S sense primer	5'-AAA/GGGIAAA/GT/CTITTT/CGAA/GTA-3'
squa/ap1-5A antisense primer	5'-GCIGTA/GTCIAA/GT/CTGT/CTC-3'
Cassette primers	
C1 primer	5'-GTACATATTGTCGTTAGAACGCGTAATACGACTCA-3'
C2 primer	5'-CGTTAGACGCGTAATACGACTCACTATAGGGAGA-3'
5' RACE primers	
R1A antisense primer	5'-AAGCAGGTCAAGGCCATGGTTCTG-3'
R2A antisense primer	5'-TTAGACACATGGAAGTGGCTGTGG-3'
3' RACE primers	
R1S sense primer	5'-CCTGGATTCCTTGACTCTCAAGG-3'
R2S sense primer	5'-TTTGGAGCAACAGCTTGACACCG-3'
MdMADS5 specific primers	
1S sense primer	5'-CAGTTTCTGGGTTGTCTTTC-3'
7A antisense primer	5'-ACTCAATATTTCTCAGGTTT-3'

Abbreviation: I, inosine.

Table 3-2. Comparison of flowering time, number of rosette leaves in T_0 transgenic and wild-type *Arabidopsis* (Col) plants in LD conditions

Transgenic line	Days to flowering [z]	Rosette leaves at time of flowering [y]	note
1	18	3	early flowering
2	16	2	"
3	16	3	"
4	20	2	"
5	20	2	"
6	28	8	
7	28	7	
8	28	8	
9	22	8	
10	28	8	
11	24	9	
12	24	10	
13	30	7	
14	28	8	
15	30	6	
wt [x]	25.5 [w]	9.5 [w]	(n=10) [v]

[z]Days to flowering is defined as the time when flower primordia were first visible to the naked eye.

[y]Rosette leaves were counted on the day that flower primordia were first visible.

[x]wt = wild-type arabidopsis columbia plants.

[w]mean number.

[v]Number of plants.

Table 3-3. Comparison of flowering time, number of rosette leaves in T_2 transgenic and wild-type *Arabidopsis* (Col) plants grown in LD and SD conditions

	LD conditions (16hr light / 8hr dark)				SD conditions (8hr light / 16hr dark)			
line	Days to flowering[z]	Rosette leaves at time of flowering[y]	Length of inflorescence (cm)[x]	No. of plants	Days to flowering[z]	Rosette leaves at time of flowering[y]	Length of inflorescence (cm)[x]	No. of plants
2-3	17.0	2.0	1.9	1	20.8±2.4	2.0±0	1.7±0.5	4
3-1	16.6±1.2	2.0±0	2.2±0.9	9	30.3±3.4	5.7±1.4	3.5±2.1	7
4-1	21.8±0.8	3.0±0.7	6.7±3.6	4	35.0±7.3	6.0±1.9	3.1±3.2	4
5-3	20.8±4.2	4.5±0.5	4.3±2.0	4	27.9±8.5	5.2±3.5	4.1±3.2	11
12-1	17.5±2.7	2.1±0.3	2.4±1.5	8	24.1±2.0	4.9±2.0	7.0±2.3	7
wt	23.6±0.5	7.3±0.5	12.6±1.7	7	63.6±1.5	18.1±3.9	-	9

[z]Days to flowering is defined as the time when flower primordia were first visible to the naked eye.

[y]Rosette leaves were counted on the day that flower primordia were first visible.

[x]Length of infloresecence was measured a month after sowing.

[z,y,x]Values are mean ± SD (standard deviation).

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                    10          20          30          40          50
MdMADS5   1   MGRGRVQLKR  IENKINRQVT  FSKRRTGLLK  KAHEISVLCD  AQVALIVFSN   50
AP1       1   MGRGRVQLKR  IENKINRQVT  FSKRRAGLLK  KAHEISVLCD  AEVALVVFSH   50
SQUA      1   MGRGKVQLKR  IENKINRQVT  FSKRRGGLLK  KAHELSVLCD  AEVALIVFSN   50

         squa/ap1-1S →
                    60          70          80          90         100
MdMADS5   51  KGKLFEYATD  SCMEQILERY  ERYSYAERQL  VEPDFESQGN  WTFEYSRLKA  100
AP1       51  KGKLFEYSTD  SCMEKILERY  ERYSYAERQL  IAPESDVNTN  WSMEYNRLKA  100
SQUA      51  KGKLFEYSTD  SCMDRILEKY  ERYSFAERQL  VSNEPQSPAN  WTLEYSKLKA  100

                              R1S →              R2S →
                   110         120         130         140         150
MdMADS5  101  KAEVLQRNHR  HYLGEDLDSL  TLKEIQNLEQ  QLDTALKQIR  LRKNQLMNES  150
AP1      101  KIELLERNQR  HYLGEDLQAM  SPKELQNLEQ  QLDTALKHIR  TRKNQLMYES  150
SQUA     101  RIELLQRNHR  HYMGEDLDSM  SLKEIQSLEQ  QLDTALKNIR  TRKNQLLYDS  150

                                                          ← R1A
                   160         170         180
MdMADS5  151  ISELQRKRKA  IQEENNLLAK  KIKEKEKAAA  QPQVQNWEQQ  N--HGLD--L  200
AP1      151  INELQKKEKA  IQEQNSMLSK  QIKEREKIL-  RAQQEQWDQQ  NQGHNMPPPL  200
SQUA     151  ISELQHKEKA  IQEQNTMLAK  KIKEKEKEIA  QQ--PQWEHH  R--HHTNASI  200

           ← R2A
                                      230         240         250
MdMADS5  201  LPQP-----  -------LPC  LNNGGT-QQD  EFLQVRRNQL  DLTLEPLYEC  250
AP1      201  PPQQHQIQHP  YMLSHQPSPF  LNMGGLYQED  DPMAM-RNDL  ELTLEPVYNC  250
SQUA     201  MPPPPQ----  YSMAPQ-FPC  INVGNTYEGE  GANEDRRNEL  DLTLDSLYSC  250

           ← squa/ap1-5A
                                      270         280         290         300
MdMADS5  251  HLGCFAA... .......... .......... .......... ..........  300
AP1      251  NLGCFAA... .......... .......... .......... ..........  300
SQUA     251  HLGCFAA... .......... .......... .......... ..........  300
```

Fig. 3-1. Comparison of the *MdMADS5* sequence with its homolog *AP1* in *Arabidopsis* and *SQUA* in *Antirrhinum*

Amino acids at positions of identity between two or three of these proteins are blocked in black. Arrows above sequences indicate primer sites.
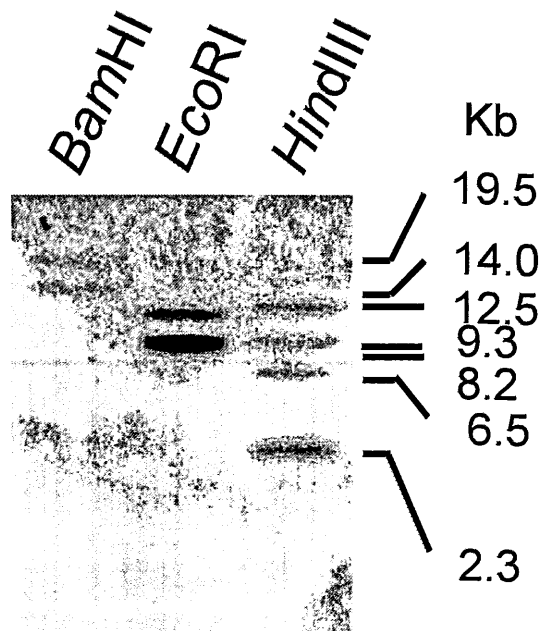
Fig. 3-2. DNA blot analysis

Equal amounts (10 μ g) of apple DNA were blotted onto a Hybond N$^+$ filter. Restriction enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII, from left to right) used to digest apple genomic DNA are indicated at the top. Hybridization was done with a DIG-labeled PCR probe lacking the MADS-box region of *MdMADS5*. The numbers to the right represent the size of the DNA in Kb.
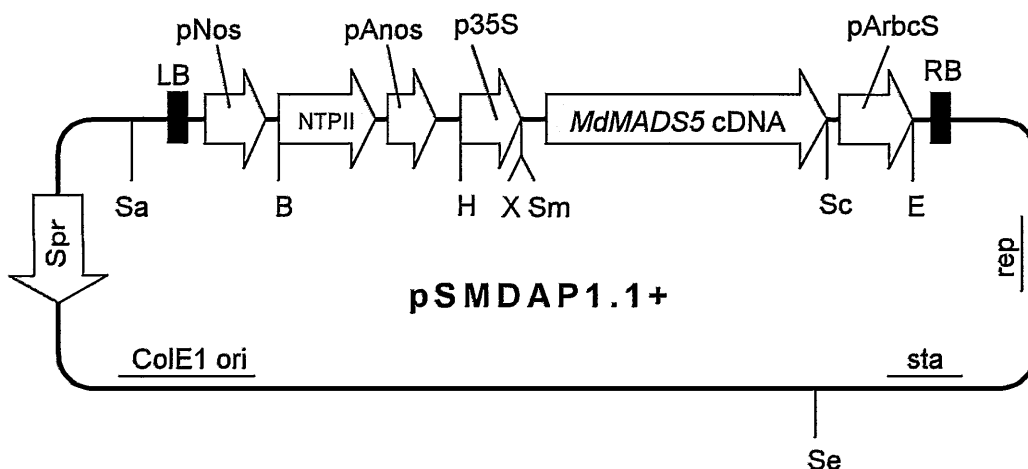
Fig. 3-3. Representation of the transformation vector pSMDAP1.1+

The *MdMADS5* cDNA was inserted in sense orientation between the *Xba*I and *Sac*I sites of the binary vector pSMAK251, giving rise to pSMDAP1.1+.

pNos, Nos promoter; pAnos, the 3' region of *nos*; p35S, cauliflower mosaic virus 35S promoter; pArbcS, the 3' region of *rbsS*; NPTII, neophosphotransferase; LB, left border; RB, right border; Sa, *Sal*I; B, *Bam*HI; H, *Hin*dIII; X, *Xba*I; Sm, *Sma*I; Sc, *Sac*I; E, *Eco*RI; Se, *Spe*I; sta, region involved in plasmid stability; rep, essential region for plasmid maintenance.
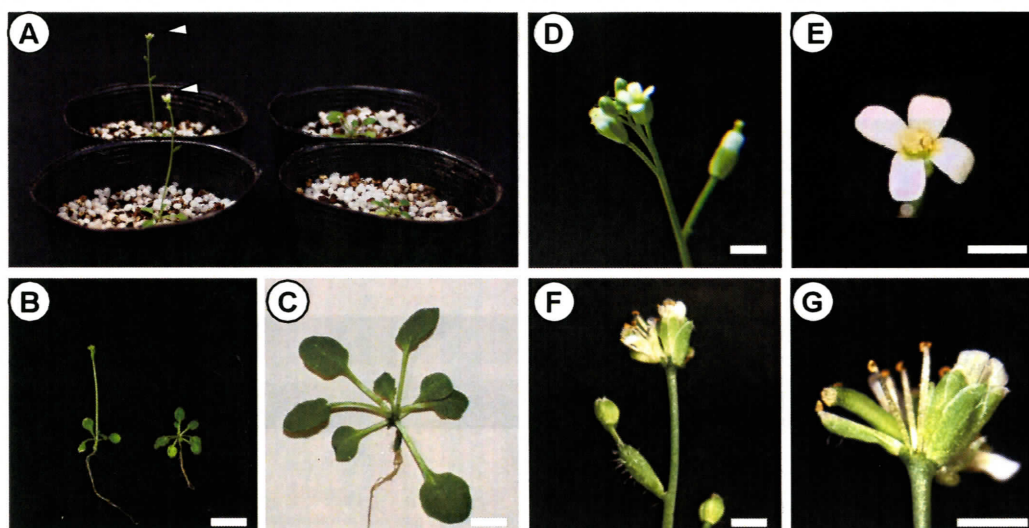
Fig. 3-4. Transgenic and wild-type *Arabidopsis* plants grown under LD photoperiods for 20 days

(A)   Transgenic *Arabidopsis* carrying a 35S::*MdMADS5* gene (left) and wild-type *Arabidopsis* Columbia (right). Arrows indicate terminal flowers.
(B)   Whole plants of transgenic (left) and wild-type (right) *Arabidopsis*.
(C)   Rosette leaves of wild-type *Arabidopsis*.
(D)   Inflorescence of wild-type *Arabidopsis*.
(E)   Close-up view of a flower of wild-type plant.
(F)   Inflorescence of transgenic *Arabidopsis*.
(G)   Close-up view of a terminal flower of the transformant.

Arabidopsis plants were grown in one-half strength of MS medium for 10 days and transferred to potting soil. Scale bar = 10 mm (B), 3 mm (C) and 2 mm (D, E, F, G). Photos were taken 20 days (A, B, C, F, G) and 30 days (D, E) after sowing.
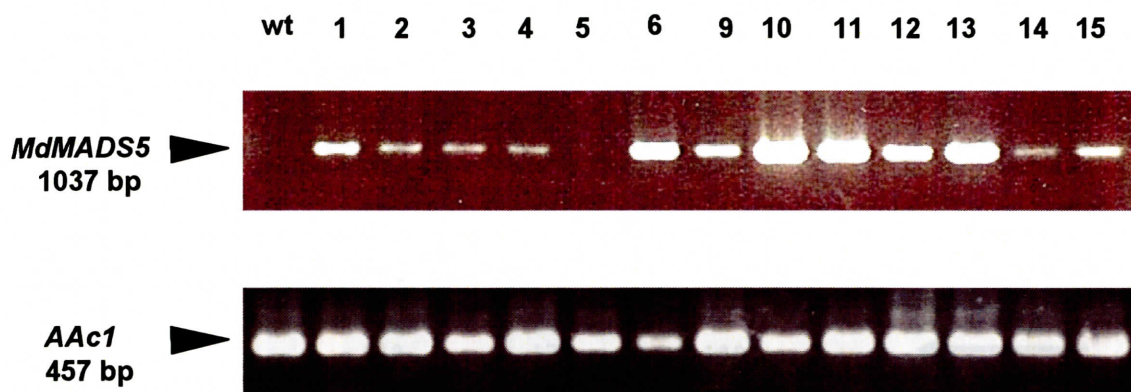
Fig. 3-5. Analysis of *MdMADS5* expression in transgenic *Arabidopsis*

Detection of *MdMADS5* was performed by RT-PCR. *MdMADS5*
was identified using 1 μ g of the total RNA as a template and the
specific primers, giving rise to a PCR product of 1037 bp. PCR
products were run on 1.5% (w/v) agarose gel stained with ethidium
bromide. The numbers above the lanes indicate the transgenic line
No. Plants of lines Nos. 7 and 8 were not examined. As an internal
control, RT-PCR on the actin *AAc1* gene was employed. wt, the
wild-type *Arabidopsis* Columbia plant.

Chapter 4

Cloning and characterization of *MdTFL1*, a *TFL1*-like gene of apple

4.1 Abstract

Unlike herbaceous plants, fruit trees such as 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 *MdTFL1* (*Malus domestica TFL1*), 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*.

4.2 Introduction

The development of all woody plants from seed includes a juvenile phase lasting up to 40 years in certain forest trees (Hacket, 1985). During the juvenile phase, flowering does not occur and cannot be induced under normal conditions (Hacket, 1985). Thus, the breeding of fruit trees

such as 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 *M. ×domestica* juvenile period lasts 7-8 years (Zimmerman, 1972), 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 apples 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* (Levy and Dean, 1998; Piñeiro and Coupland, 1998). Three *Arabidopsis* genes that control flowering time have been isolated, namely *LEAFY* (*LFY*) (Weigel al., 1992), *APETALA1* (*AP1*) (Mandel et al, 1992) and *TERMINAL FLOWER 1* (*TFL1*) (Bradley, 1997; Ohshima et al., 1997). In transgenic *Arabidopsis*, over-expression of *LFY* or *AP1* shortens the juvenile period and causes early flowering (Weigel and Nilsson, 1995) whereas *TFL1* over-expression causes late flowering (Ratcliffe et al., 1998). *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 (Ratcliffe et al., 1998; Liljegren et al., 1999).

Juvenility in fruit trees remains largely undefined. As such, there is keen interest in regulatory genes such as *TFL1, Antirrhinum CENTRORADIALIS* (*CEN*) (Bradley et al., 1996) and tomato *SELF-PRUNING* (*SP*) (Pnueli et al., 1998) that maintain the vegetative phase and promote the emergence of shoots rather than flowers. There is also strong interest in the genes *LFY* and *AP1* that promote flowering. *CEN, TFL1* and *SP* are closely related and belong to a small gene family (*CETS*) that encodes ~23-kDa proteins. These three plant genes were the first to be assigned biological functions (Pnueli et al., 2001) 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* (Shoentgen and Jolles, 1995). *FLOWERING LOCUS T* (*FT*) also belongs to the *CETS* family and is a homologue of *TFL1* (Kardailsky et al., 1999; Kobayashi et al., 1999). However, *FT* antagonizes *TFL1* function and thereby promotes flowering in concert with *LFY* (Kardailsky et al., 1999; Kobayashi et al., 1999).

In mammals, PEBPs are believed to be precursors of hippocampal neurostimulatory peptide (HCNP), and PEBPs are inhibitors of Raf-1 kinase activity (Yeung et al., 1999). 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 (Pnueli et al., 2001). Tomato SIPs bind to CETS proteins such as *Antirrhinum* CEN as well as *Arabidopsis* TFL1 and its functional antagonist FT, providing evidence that SP/SIPs interactions in tomato are conserved in distantly related plants (Pnueli et al., 2001).

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 (Kotoda et al., 2000; Sung et al., 1999; Yao et al., 1999). *MdMADS2* and *MdMADS5* promote flowering in transgenic tobacco and *Arabidopsis*, respectively, and they function analogously to *AP1* (Kotoda et al., 2002; Sung et al., 1999). 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* (Wada et al., 2002).

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.

4.3 Materials and methods

4.3.1 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 Research Center at The Ohio State University and wild-type plants were employed for *Agrobacterium*-mediated transformation. *Arabidopsis* seeds were stratified for 3 to 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).

4.3.2 Gene cloning

Full-length *MdTFL1* cDNA was obtained by the 5' and 3' rapid amplified cDNA ends (RACE) method (Chenchik et al., 1996). 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 4-1. 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 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).

4.3.3 DNA blot analysis

Genomic DNA was obtained from young Jonathan apple leaves. The DNA (10 µg) was digested individually with either *Bam*HI, *Eco*RI, *Hind*III, *Nco*I, *Xba*I, or *Xho*I according to the manufacturer's instructions (Takara Biomedicals), 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 0.5 M Na-Pi buffer (0.25 M Na$_2$HPO$_4$, pH 7.2) containing 7% SDS and 1 mM EDTA at 65 °C for 16 h followed by three washes in 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).

4.3.4 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'-GTG GCA TAC ATT GTA AAT A-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 3-1), and a sense primer 6S (5'-CAG AGG GAG CAC CCG TTC ATT GTG AC-3') and antisense primers AFL1R (5'-TTC ATT CAG TGT GCC CTA GCC-3') for *AFL1* and a sense primer 6S and an antisense primer AFL2R (5'-TCA AAC TCT CTC TGC AGA ACT GGC-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, then blotted on the Hybond-N+ (Amersham Pharmacia Biotech). Hybridizations were 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 LAS-1000 image analyzer (Fuji Photo Film).

4.3.5 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*I 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 (Yamashita et al., 1995) cut with the same restriction enzymes. The resulting plasmid was named pSMDTFL12.1.2+ (35S::*MdTFL* 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).

4.3.6 Transformation of *Arabidopsis*

A. tumefaciens strain EHA101 was used to transform *Arabidopsis thaliana* (Col) plants by the floral-dip method (Clough and Bent, 1998). For the selection of transformed plants, sterilized seeds were suspended in 0.1% sterile agar, plated on kanamycin selection plates, then transferred to a growth chamber (BIOTRON, Nippon Medical and Chemical Instruments Co., Ltd., Tokyo, Japan) set at 22 ℃ under LD conditions. The selection plates contained $0.5 \times$ MS medium (Wako Pure Chemicals Co., Ltd.), 0.8% agar (Difco Laboratories, Detroit, MI, US), 500 mg・L^{-1} Cefotaxime (Wako Pure Chemicals Co., Ltd.) and 25 mg・L^{-1} 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).

4.4 Results

4.4.1 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. 4-1 and Table 4-1, respectively.

The cDNA and predicted amino acid sequences for *MdTFL1* are shown in Fig. 4-1. The

MdTFL1 cDNA coding region exhibits 76%, 73%, 71% and 58% sequence identity to *TFL1*,

CEN, *SP* and *FT*, respectively (Fig. 4-2). 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. 4-2). The gene was therefore designated *MdTFL1* for *Malus domestica TFL1*

homologue. 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. 4-3). 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. 4-4).

4.4.2 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. Total RNA was

isolated from reproductive tissues (sepals, petals, stamens, carpels and floral buds) 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, roots and apical buds, but not in reproductive tissues such as floral organs

(Fig. 4-5). To elucidate seasonal changes in *MdTFL1* mRNA expression in the apical buds, apices

were collected from the current apple shoots during 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. 4-6). 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. 4-6, 6.19V). In

contrast, the expression of *AFL1*, an apple orthologue of *FLO/LFY* (Wada et al., 2002), was detected when the expression level of *MdTFL1* was relatively low (Fig. 4-6). On the other hand, the expression of *AFL2*, another apple orthologue of *FLO/LFY*, was detected constantly during flower development as previously reported (Wada et al., 2002).

4.4.3 *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 seven 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 4-2. 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 wt control plants under LD photoperiods (Table 4-2). 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 wt and *tfl1-1* control plants, respectively). At 35 days after sowing, a typical 35S::*MdTFL1* transformant had produced twelve to fifteen rosette leaves but no flower buds or bolting (Fig. 4-7B). In contrast, the wt plants displayed many flowers and brown pods on day 35 (Fig. 4-7A). In addition, there was little difference between wild-type *Arabidopsis* control plants and those transformed with an 35S::*MdTFL* antisense construct with respect to days to flowering and number of rosette leaves at flowering (data not shown).

4.4.4 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 (T-S10-2; Fig. 4-7D) did not flower until day 90. The 35S::*TFL1* transformant (Fig. 4-7C, right side) had not yet flowered whereas the wt control plant (Fig. 4-7C, 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. 4-7E, 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 4-2).

4.5 Discussion

Apple *MdTFL1* exhibits a high degree of sequence homology to *TFL1*, a member of the *CETS* family. Comparison of MdTFL1 with other CETS family members shows that it groups with TFL1 and four *Brassica* proteins (Fig. 4-3). It is logical that MdTFL1 groups with proteins from dicots rather than monocots because apple belongs to Rosaceae family that consists of dicotyledonous plants. Recent works revealed that *CEN/TFL1/SP* and *FT* are members of a small gene family. There are six members in *Arabidopsis* and approximately six in tomato (Carmel-Goren et al., 2003; Mimida et al., 2001). In pea, two *TFL1*-like genes exist and function differently (Foucher et al., 2003). Poplar, which is a woody plant like apple, contains at least eight *CETS* genes in 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* was isolated, which will be a member of

CETS family in apple. Several regions of notable sequence homology have been described for mammalian PEBPs. They include 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). 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 and carpels. These observations demonstrate that *MdTFL1* is expressed preferentially in vegetative tissues, although the expression was very weak in floral buds before flowering. *MdTFL1* is not expressed in mature leaves like *TFL1* (Bradley et al., 1997; Ratcliffe et al., 1999). In *Antirrhinum*, however, *CEN* mRNA is present not only in the apical meristem but in other tissues (Bradley et al., 1996), and tomato *SP* is expressed throughout development in all the primordial organs (Pnueli et al., 1998). Unlike *MdTFL1*, other *CETS* family genes such as *LpTFL1* in ryegrass, *RCN2* in rice and *PsTFL1* genes in pea are expressed in both vegetative and reproductive tissues (Foucher et al., 2003; Jensen et al., 2002; Nakagawa et al., 2002). *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 (Carmel-Goren et al., 2003). 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. 4-6). *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 (Buban and Faust, 1982; Foster et al., 2003; Kotoda et al., 2000). 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* (Ratcliffe et al., 1999; Amaya et al., 1999). Interestingly, the seasonal expression level of *MdTFL1* in apple apices appears to be opposite to that of *AFL1*, which gradually increases from early-July to late August, although *AFL2* expression is constant during flower development (Fig. 4-6). 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 reproductive phase in transgenic *Arabidopsis* plants (Fig. 4-7 and Table 4-2). 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 due to the similar expression pattern between *MdTFL1* and *TFL1* (Figs. 4-5, 4-6, and 4-7).

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 reproductive phase have not yet been determined. Our work shows that *MdTFL1* is a member of *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 earlier extremely (Kotoda et al., 2003). 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.

Table 4-1. Primer sequences used in PCR cloning of *MdTFL1*

Primer	Oligonucleotide
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'

Abbreviation: I, inosine.

Table 4-2. Phenotype of T_2 transformed *Arabidopsis* with *MdTFL1* sense gene

Controls and transgenic lines[z]	Days to flowering[y,w]	Rosette leaves at flowering[x,w]	Cauline leaves at flowering[x,w]	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

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

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

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

[w]All values are means ± SD (standard deviation).

```
                2S                    CCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTTAAA
   1  ATGAAAAGAGCCTCGGAGCCTCTGGTTGTTGGGAGAGTGATAGGAGATGTTCTTGATTCC    60
   1   M   K   R   A   S   E   P   L   V   V   G   R   V   I   G   D   V   L   D   S    20

  61  TTCACTGCAACAACAAAAATGTCTGTCACTTACAACACCAAGCTAGTCTGCAATGGACTT   120
  21   F   T   A   T   T   K   M   S   V   T   Y   N   T   K   L   V   C   N   G   L    40

 121  GAGCTCTTTCCTTCTGTTGTCACAGCCAAACCTAGAGTTGAGATTCAAGGAGGGGATATG   180
  41   E   L   F   P   S   V   V   T   A   K   P   R   V   E   I   Q   G   G   D   M    60

 181  AGATCTTTCTTTACTTTGGTGATGACCGACCCAGATTTTCCTGGCCCTAGTGATCCTTAT   240
  61   R   S   F   F   T   L   V   M   T   D   P   D   F   P   G   P   S   D   P   Y    80
                                       5S                      R1S

 241  CTAAGGGAGCACCTGCACTGGATTGTGACAGACATTCCAGGCACCACAGATGCCACATTT   300
  81   L   R   E   H   L   H   W   I   V   T   D   I   P   G   T   T   D   A   T   F   100
                         R2S

 301  GGAAGAGAGGTGGTGAGTTATGAGATGCCGAAGCCCAACATTGGCATCCACCGGTTTGTG   360
 101   G   R   E   V   V   S   Y   E   M   P   K   P   N   I   G   I   H   R   F   V   120

 361  TTTGTTCTTTTCAAGCAGAATCAAAGACAATCAATCAACACACCTTCCTCGAGGGATCAC   420
 121   F   V   L   F   K   Q   N   Q   R   Q   S   I   N   T   P   S   S   R   D   H   140
          R1A                              R2A

 421  TTCAGCACTCGAAGCTTCGCGGCTGAAAATGACCTGGGTCTTCCTGTCGCTGCCGTCTAC   480
 141   F   S   T   R   S   F   A   A   E   N   D   L   G   L   P   V   A   A   V   Y   160
          3A

 481  TTCAACGCGCAGAGAGAAACTGCAGCTAGAAGACGCTAGCTAGTAGCTCTACCCAGAACT   540
 161   F   N   A   Q   R   E   T   A   A   R   R   *                               172

 541  CCTCCATCCATTATCCATATATATGTTAAATAAAGGCTTCTTTAGAGATAGGCCATTGTA   600
                                                            2A
 601  ACTTTTGTTTCCCAATAACCTAAATTTTAACTTATTGACATGTGAGAAAATAAGTAACAC   660
 661  GTTATTAATTATTTACAATGTATGCCACAATATTAATTATGTTAAATTAATTATTATTAC   720
 721  CAAAAATAATTAT                                                 733
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Fig. 4-1. 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.

Fig. 4-2. Comparison of the amino acid sequences of MdTFL1 and MdFT with other CETS proteins

The proteins (translated from the cDNA sequence where necessary) are ATC (Mimida et al., 2001), BFT (Kobayashi et al., 1999), BNTFL1-1, BNTFL1-3, BRTFL1-1, and BNTFL1-2 (Mimida et al., 1999), CEN(accession no. S81193), CET1, CET2 and CET4 (Amaya et al., 1999), CiFT (accession no. AB027456), FT (accession no. AB027504), Hd3a (accession no. AB052944), LpTFL1 (Jensen et al., 2002), MdTFL1 and MdFT (accession nos. AB052994 and AB161112), MFT (Kobayashi et al., 1999), PnFT1b, PnFT2a, and PnFTL1a (accession nos. AB109804, AB110009 and AB161110), RCN1, RCN2 and RCN3 (Nakagawa et al., 2002), SP (accession no. U84140), TFL1 (accession no. U77674), TSF (Kobayashi et al., 1999), Vitis TFL1 (*Vitis vinifera* TFL1-like protein, accession no. AF378127). Identical residues at each position are shown in black.

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Fig. 4-3. Phylogenic tree of MdTFL1, MdFT and other CETS family protein sequences in Fig. 4-2

The ClustalW program was used to align 28 complete protein sequences. Bold characters represent apple proteins.

Fig. 4-4. 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 *MdTFL* DNA probe. Restriction enzymes used to digest apple DNA are shown at the top: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N, *Nco*I; Xb, *Xba*I; Xh, *Xho*I. Numbers to the left indicate DNA size markers in kbp.

Fig. 4-5. RT-PCR analysis for *MdTFL1* in various organs of apple

1 μg of total RNA was used for RT-PCR. Subsequent southern blots were performed using DIG-labeled DNA probes for *MdTFL1*. 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, Carpels, Floral buds. Vegetative tissues: Apical bud (V), Cotyledons, Stems, Roots, Apical buds. V, vegetative shoots.

Fig. 4-6. RT-PCR analysis in apical buds of apple throughout the year

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.

Fig. 4-7. Comparison of 35S::*MdTFL1* and 35S::*TFL1* transgenic *Arabidopsis* plants

(A), (B), 35-day old plants (ecotype Columbia) grown under LD conditions (16h 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) 42 days after sowing. (D) 35S::*MdTFL1* transformant (line S10-2) 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.

Chapter 5

Analysis of transgenic 'Orin' apple expressing *MdTFL1* antisense gene

5.1 Abstract

Fruit trees such as apple (*Malus* × *domestica* Borkh.) flower and set fruit only after an extended juvenile phase lasting several years. Therefore, the long juvenile phase has been limiting the efficient breeding of fruit trees. We previously suggested that *MdTFL1* (*Malus* × *domestica* *TFL1*) functions analogously to *TERMINAL FLOWER 1* (*TFL1*), and that *MdTFL1* is involved in the maintenance of the juvenile/vegetative phase in apple. To clear the function of *MdTFL1* in apple, we produced transgenic apples expressing *MdTFL1* antisense RNA. One of them flowered only 8 months after grafting to rootstocks, whereas the non-transformed control plants have not flowered in less than 6 years. As expected, the expression of the endogenous *MdTFL1* was suppressed in the transgenic lines that showed precocious flowering. In addition, the expression level of the transgene is correlated with the reduction of the juvenile phase. These findings confirm that *MdTFL1* functions like *TFL1* and that *MdTFL1* maintain the juvenile and vegetative phase in apple. Flower organs of the transgenic apples were normal in appearance, and a precocious flowering transgenic line set fruits and seeds. Interestingly, some flowers of the transgenic apple developed without undergoing dormancy. The expression of *MdTFL1* in apple may affect flower development as well as flower induction.

In the development of all woody plants from seed, there is a so-called juvenile phase, lasting up to 30 to 40 years in certain forest trees, during which flowering does not occur and cannot be induced under normal conditions (Hackett, 1985). In the fruit industry, it is important to accelerate flowering by reducing the juvenile or vegetative phase of the trees after planting in order to facilitate the earliest possible production of fruit. Breeding fruit trees such as 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. For example, the 'Fuji' apple, one of the most popular apple cultivars in the world (O'Rourke et al., 2003), first set fruit 12 years after being sowed, and it was 23 years before it was released as a cultivar (Sadamori et al., 1963). Generally, the juvenile phase in apple lasts 6 to 12 years (Visser, 1964; Zimmerman, 1972).

In fruit breeding, various practical techniques have been considered to accelerate the flowering and fruiting of seedlings. The basic idea is to grow the seedlings rapidly from the germination stage to the transition to flowering (Aldwinckle, 1975; Visser, 1964). In apple, grafting the seedlings onto dwarfing rootstocks such as 'Malling 9' ('M.9') and 'Malling 27' ('M.27') will usually bring earlier flowering by 1 or 2 years (Visser, 1964, 1973). Grafting seedlings onto bearing trees (top grafting), ringing, scoring, bark inversion, root pruning, and spraying with growth retardants may stimulate earlier flowering when applied to older seedlings, but in these conditions, the juvenile phase probably has already passed, and the seedlings are in transition (Kender, 1974; Zimmerman, 1972). Based on our experience, it would, however, be difficult to reduce the juvenile phase of apple seedlings to less than 4 years under normal growing conditions even if they are grafted on the dwarfing rootstock. The length of the juvenile phase is influenced more by genetic factors than by environmental factors. Significant correlations have

been found between the length of the juvenile phase and parent characteristics such as growth rate and the length of the vegetative phase (Lavi et al., 1992; Visser, 1965).

The most striking advances in our understanding of the genetic control of the timing of flowering have come from the study of *Arabidopsis [Arabidopsis thaliana* (L.) Heynh.] (Levy and Dean, 1998; Piñeiro and Coupland, 1998), and several genes such as *LEAFY* (*LFY*), *APETALA1* (*AP1*), *TERMINAL FLOWER 1* (*TFL1*), and *FLOWERING LOCUS T* (*FT*), which control flowering time, have been isolated from *Arabidopsis* (Bradley et al., 1997; Kardailski et al., 1999; Kobayashi et al., 1999; Mandel et al., 1992; Ohshima et al., 1997; Weigel et al., 1992). Over-expression of *LFY*, *AP1*, or *FT* shortens the juvenile phase, causing early flowering (Kardailski et al., 1999; Kobayashi et al., 1999; Mandel et al., 1995; Weigel and Nilsson, 1995), whereas over-expression of *TFL1* causes late flowering in transgenic *Arabidopsis* (Ratcliffe et al., 1998). *TFL1* plays a key role in the maintenance of the inflorescence meristem by preventing the expression of *LFY* and *AP1* (Ratcliffe et al., 1999). Apple orthologues of several of these genes such as *AFL1*, *AFL2*, *MdAP1* (*MdMADS5*), and *MdTFL1* have been isolated and characterized (Kotoda et al, 2000, 2002; Kotoda and Wada, 2005; Sung et al., 1999; Wada et al., 2002; Yao et al., 1999).

With the goal of elucidating the long juvenility of fruit trees, we are interested in regulatory genes, such as *Arabidopsis TFL1*, snapdragon (*Antirrhinum majus* L.) *CENTRORADIALIS* (*CEN*), and tomato (*Lycopersicum esculentum* Mill.) *SELF-PRUNING* (*SP*) genes, which maintain the vegetative phase and promote the emergence of shoots rather than flowers (Bradley et al., 1996; Pnueli et al., 1998), as well as genes such as *LFY* and *AP1*, which promote flowering. Therefore, we isolated and characterized the apple gene *MdTFL1*, which is homologous to *TFL1*, and concluded that *MdTFL1* functions like *TFL1* and is involved in the maintenance of juvenility in apple (Kotoda and Wada, 2005). *TFL1/CEN/SP* are closely related members of the *CETS* family, a small gene family that encodes 23-kD proteins, and they share

sequence similarity with a group of mammalian phosphatidylethanolamine-binding proteins (PEPBs) (Bradley et al., 1996, 1997; Oshima et al., 1997; Pnueli et al., 1998). The *FT* gene also belongs to the *CETS* family, but it mediates signals for flowering antagonistically with its homologous gene, *TFL1*, and promotes flowering with *LFY* (Kardailsky et al., 1999; Kobayashi et al., 1999). In other species, a number of *TFL1* homologues from tobacco (*Nicotiana tabacum* L.), oilseed rape (*Brassica napus* L.), cabbage (*Brassica oleracea* L.), ryegrass (*Lolium perenne* L.), rice (*Oryza sativa* L.), pea (*Pisum sativum* L.), and navel orange (*Citrus sinensis* L. Osbeck) have been cloned and characterized (Amaya et al., 1999; Fabrice et al., 2003; Jensen et al., 2001; Mimida et al., 1999; Nakagawa et al., 2002; Pillitteri et al., 2004).

We describe here the precocious flowering of transgenic apple trees caused by the suppression of *MdTFL1* in the first report on the extreme reduction of the juvenile phase in deciduous fruit trees.

5.3 Materials and methods

5.3.1 Plant materials

Micropropagated tissues of apple (*Malus × domestica* Borkh.) cultivar 'Orin' were used for *Agrobacterium*-mediated transformation. 'Orin' were cultured in proliferation medium [Murashige-Skoog (MS) medium containing B5 vitamin (1.0 mg · L^{-1} nicotinic acid, 10 mg · L^{-1} thiamine chloride, 1.0 mg · L^{-1} pyridoxin and 100 mg · L^{-1} myo-inositol), 1 mg · L^{-1} 6-benzyl-aminopurine (BA), 0.1 mg · L^{-1} indole-3-butyric acid (IBA), 3% sucrose, and 0.7% agar (pH5.8)] at 23-25 ℃ under long-days (LD) conditions (16 h light/ 8 h dark). Shoots were subcultured to new medium every four weeks. For northern analysis, apple leaves were collected from the experimental field or green houses at our research center in Morioka, Japan. For RT-PCR

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analysis, micropropagated tissues were used. Transformed and non-transformed shoots of tissue culture were grafted to apple seedlings or 'JM8', a progeny of crossing between 'Marubakaido' (*Malus prunifolia* ringo Asami) and 'M. 9', which was released from the National Institute of Fruit Tree Science, Japan as an easy-cutting apple dwarfing rootstock (Soejima et al., 1998). Grafted apples were grown in the isolated green house set at 20-25 ℃ during growing season. Liquid fertilizer was sprayed once a month.

5.3.2 Construction of transformation vectors

The plasmid vector pUMDTFL1- (Kotoda and Wada, 2005) was cut with *Xba*I and *Sac*I, after which the released *MdTFL1* cDNA fragment was ligated to the binary vector pSMAK251 cut with the same restriction enzymes, in an antisense-oriented manner. The product of the ligation was named pSMDTFL1.1- (35S::*MdTFL1* antisense construct). The *MdTFL1* antisense gene was driven by the cauliflower mosaic virus (CaMV) 35S promoter and the vector conferred kanamycin resistance (Fig. 5-1).

5.3.3 Transformation of apple

Apple cultivar 'Orin' was transformed with *Agrobacterium tumefaciens* strain EHA101 bearing pSMDTFL1.1-. *A. tumefaciens* was cultured overnight on a shaker in 20 ml liquid ϕB medium with 100 mg・L^{-1} trobicin at 28 ℃. After centrifugation (2000 g_n, 5 min), the pellet was resuspended in MS medium with 3% (w/v) sucrose and 150 µM acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone; Acros organics, NJ, USA) and further diluted with this medium until an optical density (OD) at 600 nm of 0.5 to 0.8 was reached. Leaf explants of apple 'Orin' were infected with the inoculum for 30 min, and then the leaf explants were transferred to co-cultivation medium [MS salt, 3.3 mg・L^{-1} thidiazuron (TDZ), 0.93 mg・L^{-1} naphtaleneacetic acid (NAA), 29.4 mg・L^{-1} acetosyringone and 0.25% gelrite]. After co-cultivation

for five to seven days at 22-25 ℃ in the dark, explants were transferred to antibiotic selection medium containing MS salt, 3.3 mg・L⁻¹ TDZ, 0.93 mg・L⁻¹ NAA, 25 mg・L⁻¹ kanamycin monosulfate, 500 mg・L⁻¹ claforan (Pharmacia and Upjohn Ltd, Tokyo, Japan) and 0.25% gelrite. When adventitious shoots appeared, cultures were transferred to antibiotic selection medium containing 50 mg・L⁻¹ kanamycin monosulfate, then incubated in the light. When shoots had produced several leaves, they were excised and transferred to proliferation medium containing 50 mg・L⁻¹ kanamycin monosulfate. After the confirmation of the transgene insertion by a PCR with specific primers for *nptII* or *MdTFL1*, putatively transformed shoots were excised and transferred to MS medium free of antibiotics.

5.3.4 DNA blot analysis

Genomic DNA was obtained from transgenic and non-transgenic 'Orin' apple leaves using CTAB-method as described by Kotoda et al. (2002). The DNA (10 µg) was digested individually with either *Bam*HI, *Eco*RI, *Hind*III, or *Xba*I according to the manufacturer's instructions (Takara Biomedicals), then separated on a 0.8% SeaKem GTG agarose gel (Cambrex Bio Sci., Rockland, ME, USA). The DNA bands were transferred to Hybond-N+ (Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridized with a digoxigenin (DIG)-labeled PCR probe encoding the *nptII* or *MdTFL1* gene (Fig 5-1). DIG-labeled DNA Molecular-Weight Marker II (Roche Diagnostics, Mannheim, Germany) was used as a size marker. Hybridization was performed in DIG Easy Hyb (Roche Diagnostics) at 42℃ for 16 h followed by two rinses in 2× SSC containing 1% SDS at room temperature for 5 min and two washes in 0.5×SSC containing 1% SDS at 68℃ for 20 min. The detections were performed according to the manufacturer's protocol (Roche Diagnostics). The resulting material was analyzed in a LAS-1000 image analyzer (Fujifilm, Tokyo, Japan).

5.3.5 RNA blot analysis

Total RNA was isolated from leaves of transgenic and non-transgenic 'Orin' apples in growing season using CTAB-method as described by kotoda et al. (2000). 20 μg of the total RNA was separated on 1.2% SeaKem GTG agarose gels (Cambrex Bio Sci.) containing 5.0% (v/v) formaldehyde. After which the gels were blotted on Hybond-N+ (Amersham Pharmacia Biotech). Hybridization was performed in DIG Easy Hyb (Roche Diagnostics, Mannheim, Germany) at 50 ℃ for 16 h with a DIG-labeled PCR probe using *MdTFL1* cDNA as a template. As an internal control, the expression of histone H3 gene was measured. The wash and detection were performed as for DNA.

5.3.6 Reverse transcription (RT)-PCR analysis

Total RNAs were isolated from cultured tissues of each transgenic lines and controls using the SV total RNA Isolation System (Promega, Madison, USA). A specific primer (5'-TTG TGG CAT ACA TTG TAA ATA-3', 21 bp) for *MdTFL1* and a random primer for *AFL1*, *AFL2*, *MdAP1*, and histone H3 gene (internal control) were used in reverse transcription reaction (30 ℃ for 10 min, 42 ℃ for 50 min, 99 ℃ for 5 min) with 1 μg of total RNA. Specific transcripts of *MdTFL1*, *AFL1*, *AFL2*, *MdAP1* and histone H3 genes were identified using gene specific primer sets shown in Table 1. Conditions of amplification for each primer sets were as follows: 1 cycle of 300 sec at 94 ℃, 40 cycles of 30 sec at 94 ℃, 30 sec at 53 ℃, 60 sec at 72 ℃ for *MdTFL1* and histone H3, 1 cycle of 300 sec at 94 ℃, 40 cycles of 30 sec at 94 ℃, 30 sec at 50 ℃, 60 sec at 72 ℃ for *AFL1* and *AFL2*, 1 cycle of 300 sec at 94 ℃, 40 cycles of 30 sec at 94 ℃, 30 sec at 55 ℃, 60 sec at 72 ℃ for *MdAP1*. Amplified products were separated on 1.5% SeaKem ME agarose gel (Cambrex Bio Sci.) in 1× TAE buffer. DNA was then stained in an ethidium bromide solutions at 1.0 μg · mL^{-1} for 20 min.

5.3.7 Pollen viability test

Anthers from the transgenic lines and control plant 'Orin' were collected in the 1.5 mL tube, and incubated at 25 ℃ with silica gel for dehiscence of anther. Thereafter, pollen was plated on the 1% agar containing 17% sucrose, and then incubated at 25 ℃ for pollen germination. The pollen germination was photographed through light microscopy (Nikon, Tokyo, Japan).

5.4 Results

5.4.1 Transformation of apple with *35S::MdTFL1* antisense gene

To clear the function of the *MdTFL1* (GenBank accession no. AB052994), a putative orthologue of *Arabidopsis TFL1*, we produced transgenic apples with the *MdTFL1* antisense gene on the purpose of suppressing the expression of the endogenous *MdTFL1*. About two thousand leaf discs of the apple cultivar 'Orin' were used in the *Agrobacterium*-mediated transformation. The kanamycin resistant shoots regenerated from five independent leaf discs were cultured in the proliferating medium after selection. The five kanamycin resistant shoots were grafted to rootstocks (Table 5-2), and then transferred to an isolated green house. Each line was multiplied by grafting individual shoots in tissue culture to 'JM8' rootstocks, giving rise to one to four clones per line. Introgression of the transgene was confirmed by a PCR with specific primers for the *nptII* and *MdTFL1*, revealing that three independent transformants (303, 614 and 705) and two escapes (837 and 850) were produced (Table 5-2).

5.4.2 DNA blot analysis of transgenic lines

DNA blot analysis was performed for transgenic lines using *nptII* (neomycin

phosphotransferase II gene) and *MdTFL1* probes to demonstrate the copy number of the transgene (Fig. 5-2). DNA blot probed with a *nptII* cDNA fragment showed that the line 614 has one copy and the lines 303 and 705 have two copies of the transgene (Fig. 5-2A). Every line has a common 2.9 kb fragment derived from the region between two *Bam*HI sites of T-DNA on the lane of *Bam*HI digestion (Fig. 5-1 and Fig. 5-2A, arrowhead). DNA blot probed with *MdTFL1* cDNA fragment also reflected the result of that with *nptII* (Fig. 5-2B).

5.4.3 Precocious flowering of transgenic apples

Transgenic line 705-1 produced a solitary flower only eight months after grafting to rootstock (Fig. 5-3A; Table 5-2), although apple seedlings flower naturally after seven to eight years (Zimmerman, 1972). Other lines also showed precocious flowering, ranging from 11 to 25 months after grafting (Table 5-2). In contrast, no flowering took place in the non-transgenic controls for about six years (Table 5-2). In this connection, not a single instance of precocious flowering has been seen in the more than 100 other transgenic lines produced with various constructs, including disease resistant genes which encode glucanase or chitinase at the National Institute of Fruit Tree Science (data not shown). After breaking of the first dormancy, six transgenic apples, including lines 303 and 705, produced some solitary flowers on their leaf axils and at the tops of two-year-old or current shoots (Fig. 5-3B and C). The normal apple trees produce many clusters comprised of about five flowers on their fruit-bearing shoots. These transgenic lines also produced clusters comprised of normal numbers of flowers from second year (Fig. 5-3B and C). As to leaf morphology, line 303 had round, more serrate leaves and line 705 had leaves with much smaller stipules than those of the controls (Fig. 5-3D).

In the line 303, the branches were relatively short and upright compared to the other lines and controls (Table 5-2). The lines 614 and 705 showed weaker growth habits as a whole, and the branching is a little more complicated than controls because foliar buds were often converted to

floral buds. The diagram of growth habits of the control and the transgenic line is shown in Fig. 4-4. The apple seedling flowers at the maturation stage after undergoing 7 to 8 years of juvenile period (Fig. 5-4A). On the other hand, the transgenic lines with *35S::MdTFL1*-antisense (AS) flowers at the very early stage of the development (Fig. 5-4B). The conversion of foliar buds (shown in brown arrowhead) into floral buds (shown in pink arrowhead) often leads to the shoot growth cessation, resulting in the change of plant architecture with shorter and complicated branches (Fig. 5-4C).

5.4.4 Flower and fruit development of the precocious flowering line.

The first flower of the most precocious line 705-1 had no stamens (Fig. 5-5A), whereas flowers after the following season were normal in appearance (Fig. 5B) like those of control 'Orin' (Fig. 5-5C). To confirm the fertility of transgenic apples that show precocious flowering, we carried out pollination between transgenic apples and other non-transgenic apple cultivars. Line 705-1, pollinated with the Japanese apple cultivar 'Sansa', set some fruits (Fig. 5-5D). On the other hand, the representative line 705-1 was used to pollinate apple cultivars 'Sansa' and 'McIntosh Wijcik' because pollen from the precocious lines (Fig. 5-5J and K) germinated on agar plates as the controls (Fig. 5-5L), resulting in setting some fruits (Fig. 5-5E). Development of transgenic fruits appeared normal (Fig. 5-5D and E) as compared to non-transgenic controls grown normally in the field (Fig. 5-5F). Fruits from the line 705-1 were harvested about six months after pollination (Fig. 5-5G). The skin color and the aroma of the transgenic fruits were almost the same as those of non-transgenic 'Orin' fruits although the size of the transgenic fruit was about one-third of that of normal fruit. Some transgenic fruits were seeded (Fig. 5-5H), and others were often seedless. Interestingly, the precocious line had a tendency to set some fruits without fertilization, suggesting that the antisense expression of *MdTFL1* may reinforce parthenocarpy in apple since the original 'Orin' apple occasionally produces parthenocarpic

seedless fruits. Several apple mutants such as 'Noblow', 'Wellington Bloomless', and 'Spencer Seedless' produce apetalous flowers that develop into parthenocarpic seedless fruit (Stout, 1929). However, flowers from the transgenic line or control 'Orin' apple had four normal floral organs (sepals, petals, stamens, and pistills) (Fig. 5-5B and C) unlike the apetalous flowers of those mutants.

5.4.5 Expression analysis of transgenic lines.

Transgene expression in the leaves of transformants was analyzed by RNA blot hybridization probed with the *MdTFL1* (Fig. 5-6A). The expression was relatively strong in transgenic lines 303-1 and 705-1 as compared to the line 614-1, correlated inversely with the time to flowering in transformants (Fig. 5-6A; Table 5-1). To detect the expression of the endogenous *MdTFL1*, reverse transcription (RT)-PCR was performed as shown in Fig. 5-6B. Total RNAs from some shoots of tissue culture in each line were used because *MdTFL1* is normally expressed in vegetative shoot apices, not in leaves (Kotoda and Wada, 2005). As expected, the expression of the endogenous *MdTFL1* was suppressed in the transgenic lines as compared to the control 'Orin' apple although it was still detectable in the transgenic lines (Fig. 5-6B). To examine the effect of the antisense suppression of *MdTFL1* on the other genes involved in flowering of apple, the expression of *AFL1*, *AFL2* and *MdAP1* (*MdMADS5*) in transgenic lines were analyzed by RT-PCR using the RNAs from shoots of tissue culture (Fig. 5-6C). *AFL1* and *AFL2* are apple orthologues of *FLORICAULA/LEAFY* (Kotoda et al., 2000; Wada et al. 2002), and *MdAP1* is an apple homologue of *APETALA1* (Kotoda et al., 2000, 2002; Yao et al., 1999). *AFL1* was up-regulated in the line 303 (Fig. 5-6C) although *AFL1* is not expressed in the vegetative tissues but specifically in floral shoot apices in normal apple (Wada et al., 2002). For *AFL2*, which is normally expressed in both vegetative and floral shoot apices, the gene expression was seen in the transgenic lines as well (Fig. 5-6C). The expression of *AFL2* was relatively high in the line 705,

which flowered the earliest of all the transformatns. The expression of *MdAP1* was not detected in both transgenic and non-transgenic lines (Fig. 5-6C). In this expression analysis, the histon H3 gene of apple was adopted as an internal control.

5.5 Discussion

There are two ways to produce plants with precocious flowering. One is the constitutive expression of genes that promote flowering, such as *LFY, AP1* and *FT*. The other is the suppression of genes that delay flowering, such as *TFL1*, by antisense expression, co-suppression, or RNA interference (RNAi). We showed here that antisense expression of *MdTFL1* effectively induces precocious flowering in transgenic apple. The transgenic lines 303 and 705 first flowered 8 to 15 months and the line 614 first flowered 25 months after grafting in contrast to the control that first flowered in 69 months (Table 5-2). The expression level of the transgene was relatively high in the lines 303 and 705 with two copies of the transgene, as compared to the line 614 with one copy (Fig. 5-2, Fig. 5-6A). These results showed that the expression level of the transgene is correlated with the precocity of the transforamants. As expected, the expression of the endogenous *MdTFL1* was suppressed in transgenic lines as compared to a control (Fig. 5-6B), indicating that *MdTFL1* intrinsically maintains the juvenile phase in apple. The knockout of the *MdTFL1* gene by co-suppression or RNAi would induce much more precocity, based on the result that the endogenous *MdTFL1* in the transformants was not suppressed completely by the antisense method. Some defects in flower organs of transgenic lines such as no pistil or decreased number of flowers per cluster would be attributed to the underdevelopment of floral buds, not directly to the function of *MdTFL1* because those defects or unusual solitary flowers didn't become observed as they grew. Most flowers of the transgenic lines had functionally normal reproductive organs,

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resulting in normal fruits that contained several seed (Fig. 5-5G and H). In fact, it was found that the line 705 had both pollen and seed viability by crossing tests. These results point out that the precocious line with *35S::MdTFL1* could be both a pollen and seed parent in cross breeding.

Floral bud differentiation in transgenic line 705 occurred several times over a period of at least four months, and flowers were produced sequentially without dormancy (Fig. 5-7A and D). In addition, line 705 produced flowers on current shoots that look like vegetative ones and grow continuously (Fig. 5-7A and B). Consequently, shoots that produce flowers ceased vegetative growth, causing the tree to become less vigorous and to develop shorter branches than the controls (Fig. 5-4C). In normal apple trees, floral bud differentiation occurs about six to eight weeks after anthesis, and flower primordia develop into floral organs by the following spring. In addition, normal apple trees neither induce floral bud differentiation on the vegetative shoot, nor come into flower even on reproductive shoots growing after anthesis in the current season (Fig. 5-7C), rather they flowered naturally the following season after breaking dormancy. Dormancy (when extension growth is prevented by physiological processes inside the plant) in deciduous fruit trees and other woody perennials of the temperate zones occurs annually, enabling plants to survive cold winters (Saure, 1985). Line 705 appears to require little dormancy for flowering and to have acquired the competence to respond to floral induction signals for several months although normally the timing of flower induction is regulated strictly by both environmental and endogenous factors. Antisense expression of *MdTFL1* frequently shortened the period of flower development, possibly by abolishing dormancy. These findings suggest that in apple *MdTFL1* affects flower development and plant architecture as well as flower induction.

We produced several transgenic apples that ectopically expressed genes possibly involved in flowering, but there were few transgenic apples that markedly induced precocious flowering other than those expressing *MdTFL1* antisense RNA. In citrus, constitutive expression of *LFY* or *AP1* produced precocious flowers 12-20 months after transfer to the green house (Peña

et al., 2001). However, it is not clear whether there is a common mechanism of flower induction in citrus and apple, because citrus, an evergreen fruit tree, is very different from apple, a deciduous fruit tree, in their growth and fruiting habits. In fact, unlike citrus, constitutive expression of *MdAP1* or *AFL* in apple has not caused precocious flowering, except for the single instance of apple that expresses *Arabidopsis AP1* (Kotoda et al., unpublished data). Therefore, the competence of apple to respond to the constitutive expression of *AP1* or *LFY*-like genes might be lower than that of citrus. In the poplar, over-expression of *PTLF* infrequently caused early flowering although competence to respond to the constitutive expression of *LFY* varies widely among *Populus* genotypes (Rottmann et al., 2000). Together with the fact that only a few instances of precocious flowering in woody plants by over-expression of *LFY* or *AP1* have been reported, *LFY* and *AP1* may not be effective factors for breaking the juvenile and/or vegetative phases of deciduous fruit trees such as apple. Probably in apple, a *CETS* family gene like *MdTFL1* has an important role in regulating the transition to flowering

Early July is the beginning of floral bud differentiation at our institute when *MdTFL1* mRNA is expressed strongly in apices of current apple shoots (Kotoda and Wada, 2005). The expression of *MdTFL1* mRNA, however, decreases gradually until mid-December paralleling the development of flower primordia. Previously we reported that the expression of *AFL1* gene was detected after the onset of floral bud differentiation and that *MdAP1* mRNA appeared concurrent with sepal formation (Kotoda et al., 2000; Kotoda and Wada, 2005; Wada et al. 2002). *AFL1* or *AFL2* was up-regulated respectively in the precocious lines 303 or 705 as compared to a control, whereas *MdAP1* was not expressed in those transgenic lines (Fig. 5-6C). Thus, down-regulation of *MdTFL1* might trigger the up-regulation of *AFL1* and/or *AFL2* in apical buds, resulting in flower induction in apple although the up-regulation of *AFL1* or *AFL2* was transient in the transgenic lines with *35S::MdTFL1*. It is interesting that the juvenile and/or vegetative phases can be regulated by only a *TFL1*-like gene although the maintenance of them is one of the most

important events in the early stage of development in woody plants. Based on these results, *MdTFL1* must be one of the factors that control transition from the vegetative to reproductive phase in apple.

Like TFL1, CEN, SP, and FT, the putative protein encoded by the *MdTFL1*-shared sequence is similar to the mammalian phosphatidylethanolamine-binding proteins (PEBP) originally named for their ability to bind phospholipids *in vitro* (Schoentgen and Jollès, 1995). In mammals, PEBP is an inhibitor of Raf-1 kinase activity (Yeung *et al.*, 1999). Several years ago, the crystal structure of CEN was determined, suggesting that the biological effects of *CEN* arise from its ability to form complexes with phosphorylated ligands (Banfield and Brady, 2000). In the Raf1 system, RKIP, an SP homologue protein from mammals, binds to Raf1 as tomato SP binds to SPAK (SP-associated kinase) in the SP system (Pnueli, 2001; Yeung et al., 1999). *MdTFL1* may also functions by interacting with proteins like SPAK or other factors involved in flowering.

In citrus, over-expression of *CiFT*, a citrus homlogue of *FT*, induces precocious flowering extremely, indicative that *CiFT* has a key role in the regulation of flower induction (T. Endo, personal communication). *FT* is a member of the *CETS* family and has conserved motifs that contribute to the conformation of the ligand-binding site, but its function is antagonistic to *TFL1*. Studies of factors that interact with *CETS* genes are needed to clarify the mechanism that underlies the transition to flowering in woody plants.

Conclusion

We have shown how effectively antisense expression of *MdTFL1* induce precocious flowering of apple, for the first time in the deciduous fruit trees. In addition, we have shown that *MdTFL1* has a key role in the regulation of juvenility, flower induction, and flower development

in apple. The reduction of generation time obtained by the use of this transgenic approach to suppress endogenous *TFL1*-like genes would be applicable not only to fruit trees but also to the other woody plants that have a long juvenile period. The future use of these techniques should be of advantage in breeding, crop production, and basic research such as molecular studies on woody plants, including fruit trees.

Table 5-1. Gene-specific primer sets used for RT-PCR analyses of transgenenic lines

Primer	Oligonucleotide
MdTFL1	
Sense primer	5'-CTC TTA AAA TGA AAA GAG CCT CGG-3' (24 mers)
Antisense primer	5'-TTG TGG CAT ACA TTG TAA ATA-3' (21 mers)
AFL1	
Sense primer	5'-GCA CCC GTT CAT TGT GAC GG-3' (20 mers)
Antisense primer	5'-GCC CAA AAT CTC TCG CCC-3' (18 mers)
AFL2	
Sense primer	5'- GCA CCC GTT CAT TGT GAC GG -3' (20 mers)
Antisense primer	5'-CTT ATC AGT TAT ATT GAA GCG-3' (21 mers)
MdAP1	
Sense primer	5'-ATG GGG AGA GGT AGA GTT CAG CTT-3' (24 mers)
Antisense primer	5'-TTA GAC ACA TGG AAG TGG CTG TGG-3' (24 mers)
Histone H3	
Sense primer	5'-TGA AGA AGC CCC ACA GAT A-3' (19 mers)
Antisense primer	5'-ACA CAA GAA ACT ATA AAC C-3' (19 mers)

Table 5-2. Phenotype of transformed 'Orin' apples with the *MdTFL1* antisense gene

Transgenic Line[z]	*nptII* gene[y]	Transgene RNA[x]	Time to first flowering[w]	Growth habit[v]	Grafted year
Control-1	ND	ND	69	N	1997
Control-2	ND	NT	-	N	2000
303-1	+	+	11	SB	2000
303-4	+	+	11	SB	2000
705-1	+	+	8	W, SB	2000
705-2	+	+	13	W, SB	2000
705-3	+	+	11	W, SB	2000
705-4	+	+	11	W, SB	2000
614-1	+	+	25	W	2001
837-1	ND	NT	-	N	2002
837-2	ND	NT	-	N	2002
850-1	ND	NT	-	N	2002
850-2	ND	NT	-	N	2002
850-3	ND	NT	-	N	2002

[z]Three independently regenerated non-transformed 'Orin' apples were grafted on seedlings (control-1) or 'JM8' rootstocks (control-2). Numbers following the line (eg. 303-1 and 303-4) mean clones multiplied by greenwood grafting of tissue cultured shoots.

[y]DNA was extracted from the leaves, and PCR was performed to detect *nptII* genes.

[x]Total RNA was extracted from the leaves, and Northern blot analysis was performed to detect the transgene RNA.

[w]Months counted from grafting and transfer to the greenhouse.

[v]N, normal growth habit; SB, short branch; W, weak tree vigor.

ND, not detected; NT, not tested

Fig. 5-1. The schematic representation of the transformation vector pSMDTFL1.1-

MdTFL1 cDNA was inserted in the antisense orientation between the *Xba*I and *Sac*I sites of the binary vector pSMAK251, giving rise to pSMDTFL1.1-. The regions of the probes to detect *npt*II or *MdTFL1* gene are indicated in the bald line above the each gene. Pnos, *nos* promoter; Tnos, the 3' region of *nos*; P35S, cauliflower mosaic virus 35S promoter; TrbcS, the 3' region of *Arabidopsis rbcS-2B* gene; *nptII*, neomycin phosphotransferase II gene; LB, left border; RB, right border; SpR, Spectinomycin/Streptmycin resistance gene from Tn7; sta, region involved in plasmid stability; rep, region essential for plasmid maintenance.

Fig. 5-2. Southern blot analyses of transformants of 'Orin' with *MdTFL1* antisense gene

Genomic DNA (10 µg) was isolated from the leaves of three transformnats and an untransformant, digested individually with either *Bam*HI , *Eco*RI, *Hind*III, or *Xba*I, then separated on a 0.8% agarose gel. The DNA bands were transferred to Hybond-N+ and hybridized with a DIG-labeled PCR probe encoding the *nptII* (A) or *MdTFL1* gene (B). DIG-labeled DNA molecular weight marker was used as a size marker. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; X, *Xba*I.

Fig. 5-3. Precocious flowering of apple by constitutive expression of the *MdTFL1* antisense gene

(A)   Eight-month-old transgenic line 705-1 grafted onto rootstock. Terminal flower (tf) and graft union (gt) are indicated by arrowheads.

(B)   Twelve-month-old transgenic line 705-1 after the breaking of the first dormancy. Solitary flower (sf) and flower cluster (fc) are indicated by arrowheads.

(C)   Eleven-month-old transgenic line 705-4.

(D)   Leaves from transgenic lines 303-1 (left), 705-1 (center), and a non-transgenic control (right).

Scale bars: (A), 10 cm; (B) and (C), 3.0 cm; (D), 2.0 cm.

Fig. 5-4. Schematic representation of phenotype of a wild-type and a transgenic apple with *35S::MdTFL1*-AS.

The expression of *35S::MdTFL1*-AS causes changes in plant architecture

(A)   Control wild-type (WT) plant. No flowers are present on vegetative shoots growing upright.
(B)   One-year-old precocious line expressing *35S::MdTFL1*-AS. Flowers are present at the top of the shoot and in leaf axils, resulting in termination of the shoot growth.
(C)   two-year-old precocious line. Replacement of shoots by flowers causes weak tree growth.

Fig. 5-5. Flowers, fruits, and pollen viability of the transformants

(A) to (C) Flowers of transgenic and control plants. (A) Close-up of the solitary flower of the transgenic plant in Fig. 3A. (B) Close-up of the flower cluster of the transgenic plant shown in Fig. 3B. (C) Close-up of flowers from a control plant grown in the field. tf and lf indicate terminal flower and lateral flower, respectively, in (B) and (C). (D) to (F) Fruits of transgenic and control plants. (D) A four-week-old young fruit of transgenic 705-1 crossed with 'Sansa'. (E) A four-week-old young fruit of 'Wijcik' crossed with line 705-1. (F) A four-week-old young fruit of the control plant. (G) to (I) Fruit development with and without pollination. (G) Mature fruits with pollination. (H) Insides of the fruits in (G). Left, fruit having a seed, right, seedless fruit. (I) Young fruits of line 705 that developed without pollination. Arrowheads indicate parthenocarpic fruits (pf). (J) to (K) Pollen germination of transgenic and control plants on 1% agar containing 17% sucrose. (J) Pollen from line 705-3. (K) Pollen from line 705-4. (L) Pollen from control plant. Scale bars: (A) to (C), 0.5 cm; (D) to (F) and (G) to (I), 2.0 cm; (J) to (L), 100µm.

Fig. 5-6. Expression analyses of transformants with the *35S::MdTFL1* antisense construct by northern blot and RT-PCR

(A)Northern blot analysis of transgene expression in the leaves of the transformants.
(B)RT-PCR analysis of the endogenous *MdTFL1* in the shoots of tissue culture.
(C)RT-PCR analysis of apple genes involved in flowering in the shoots of tissue culture.

In the expression analyses, untransformed 'Orin' and histone H3 were used as a control plant and an internal control gene, respectively. EtBr, ethidium bromide; PC, positive control (plasmid containing each gene); *AFL1* and *AFL2*, apple homologues of *FLORICULA/LEAFY*; *MdAP1*, an apple homologue of *APETALA*.

Fig. 5-7. Ever-flowering characteristic of the transformants with *35S::MdTFL1*-antisense (AS)

(A) Young fruits (yf) and flower buds of transformants developed simultaneously. The flower bud was produced at the top of the current shoot. (B) Close-up of the flower bud (fb) shown in (A). (C) and (D) The schematic drawing of transgenic and control wild-type (WT) plants. The transgenic apple with *35S::MdTFL1*-AS appears to have a defect in dormancy, resulting in an ever-flowering character in contrast to the control plant. (C) Floral bud (shown in pink arrowhead) differentiation normally occurs after the cessation of shoot growth. Extension growth of shoots is prevented by dormancy in the current year. (D) Floral bud differentiation occurs on the growing shoot (shown in green), and flowers are produced without dormancy in the transgenic precocious line.

Chapter 6

General discussion

From the observation that growth of most current shoots from the cluster base of 'Jonathan' apple ceased in late June in Morioka, Japan, the transition from the vegetative to reproductive phase in 'Jonathan' apple appears to occur in late June in Morioka. However, the morphological changes in the apical meristem were not detectable in the microscopic examination at the time of the phase transition. Several genetic pathways for floral bud differentiation would be activated dramatically within about two weeks after the phase transition, resulting in the morphological changes in the apical meristems. In apple, phase changes from vegetative to reproductive growth must be studied morphologically, physiologically, and genetically in detail during the period between late June (5 WAFB) and mid July (8 WAFB).

Based on the sequence analysis, *MdAP1* (*MdMADS5*) was classified into the *Arabidopsis AP1/CAL* group (Fig. 6-1). In addition, transgenic *Arabidopsis* ectopically expressing *MdAP1* flowered 5 to 10 days earlier than wild-type plants. However, *MdAP1* mRNA was not detected in the floral meristem until mid October when the sepal primordia were differentiated. These results suggested that *MdAP1* was not directly involved in floral induction. *MdAP1* would function in the formation of sepals and receptacles where it was expressed preferentially. In apple, sepals and receptacles are the organs which will develop into edible parts or fruits. Considering that *MdAP1* (*MdMADS5*) was expressed in the skin and cortex of apple (Yao et al., 1999), it might function in the development of fruits in apple. Recently, we have shown that *MdAP1* complemented the phenotype of *Arabidopsis ap1-1* loss-of-function mutant (Kotoda et

al., unpublished result). To clear the functions of *MdAP1*, both loss and gain-of-function experiments using transgenic apples will be required. Because *MdMADS2* and *MdMADS12* are also classified into *AP1* group, those *MADS*-box genes are to be analyzed as well (Fig. 6-1).

Apple *MdTFL1* exhibits a high degree of sequence homology to *TFL1*, a member of *CETS* family. *MdTFL1* is possibly involved in the regulation of flower induction from late June to early July (6-8 WAFB) since *MdTFL1* is expressed preferentially in apical buds of vegetative shoots and expressed strongly in early July, approximately 2 weeks prior to the initiation of floral bud formation, with gradual decrease to late July. It was interesting that the seasonal expression level of *MdTFL1* in apple apices appears to be complementary to that of *AFL1*. In the transgenic apples expressing *MdTFL1* antisese RNA, *AFL1* or *AFL2* was transiently up-regulated, suggesting that *AFL1* and/or *AFL2* are negatively regulated by *MdTFL1*. The genetic interaction of *MdTFL1* and *AFL1/AFL2* remains to be analyzed in detail. One of the transgenic 'Orin' apples expressing *MdTFL1* antisense RNA flowered only 8 months after the transfer to the greenhouse, demonstrating that antisense expression of *MdTFL1* reduces the juvenile phase in apple. The expression of endogenous *MdTFL1* was suppressed in the precocious lines, confirming that decrease of the endogenous *MdTFL1* mRNA induced precocious flowering. Recently, another gene homologous to *MdTFL1* and two apple genes classified into *CEN* sub-group have been isolated and characterized (Naozumi Mimida et al., unpublished results). In pea, different *TFL1* homologues control two distinct aspects of plant development; *PsTFL1a* (*DET*) acts to maintain the indeterminancy of the apical meristem during flowering, whereas *PsTFL1c* (*LF*) delays the induction of flowering by lengthening the vegetative phase (Foucher et al., 2003), on the contrary to *Arabidopsis TFL1*, which performs both functions in *Arabidopsis.*

Thus, the functions of apple *TFL1/CEN* homologues besides *MdTFL1* must be cleared.

The length of juvenile phase is inversely related to the breeding efficiency of woody perennials such as fruit trees. Also, the genetic and molecular studies in fruit trees fall behind the herbaceous model plants such as *Arabidopsis*, rice and tomato because of their long juvenile phase. As for the reduction of generation time of apple, several F_1 seedlings have actually been obtained from the compatible cross between 'Sansa' and the transgenic precocious line 705 in this study (data not shown). In apple, gain-of-function and loss-of-function experiments became possible since the transformation system was first developed by James et al. (1989). However, the long juvenile period has hindered the early analysis of gene functions in reproductive tissues of the transgenic apples for many years. Therefore, the transgenic approach to suppress endogenous *TFL1*-like genes will hasten the flowering and fruiting of the transgenic apple and enable us to analyze the function of the genes or the activity of the gene promoters in reproductive organs within a few years after regeneration. Crossing between precocious transgenic apples and non-transgenic apple with agronomically important traits (AIT) such as disease resistance will produce precocious apple seedlings with those good traits. After several crossings of the precocious lines with the non-transgenic lines having AIT, we could select some segregated lines with no transgenes (Fig. 6-2). Breeding strategy like this will moderate the social problems on the public acceptance of genetically modified crops. To facilitate the function analysis of genes expressed in apple fruits, co-expression system and re-introduction system have been postulated (Fig. 6-3A). In the co-expression system, both a flowering-promoting gene cassette and a target gene cassette reside on the same vector (Fig. 6-3B). In the re-introduction system, a target gene is introduced into precocious flowering apples such as line 705. Using these systems, we could analyze the gene

function in fruits within one or two years after producing transgenic plants. The future use of these techniques should be of advantage in breeding, crop production, and basic research such as molecular studies on woody plants, including fruit trees.

General conclusion

1. In 'Jonathan' apple, the transition from the vegetative to reproductive phase occurred in late June [about 6 weeks after full-bloom (WAFB)] when the shoot growth ceased, and floral bud differentiation was initiated from mid July (about 8 WAFB) in Morioka, Japan.

2. *AFL* (Apple *FLORICAULA/LEAFY*) was considered to be involved in the floral induction in apple because it was highly expressed in the apices around the stage of the floral bud differentiation. [Later, it was found that there were two apple homologues of *FLORICAULA/LEAFY*, *AFL1* and *AFL2*, and that *AFL1* was expressed specifically in the apices of floral buds, whereas *AFL1* was expressed constantly in various organs (Wada et al., 2002).]

3. *MdAP1* (*MdMADS5*) was classified into *AP1* group by the sequence analysis. It was expressed specifically in sepals concurrent with sepal formation. Transgenic *Arabidopsis* expressing *MdAP1* flowered 5-10 days earlier than control wild-type plants. Base on these results, *MdAP1* was considered to be an apple orthologue of *Arabidopsis AP1*.

4. *MdTFL1* was grouped with the *TFL1* subgroup of dicots by sequence analysis. It was expressed preferentially in vegetative tissues of apple seedlings and the expression was peaked about 2 weeks prior to floral bud differentiation. Transgenic *Arabidopsis* expressing *MdTFL1* flowered much later than control wild-type plants. These results suggest that *MdTFL1* is involved in the maintenance of the juvenile/vegetative phase in apple and that it functions analogously to *Arabidopsis TFL1*.

5. The transgenic apple expressing *MdTFL1* antisense gene flowered only 8 months after grafting to rootstocks in contrast to the non-transformed control 'Orin' apple which did not flower in less than 6 years. This precocious flowering phenotype of the transgenic apples was due to the suppression of the endogenous *MdTFL1* gene expression. Based on these results, it was found that *MdTFL1* intrinsically maintain the juvenile/vegetative phase in apple.

6. Some flowers of the transgenic apple developed without undergoing dormancy and showed an ever-flowering characteristic, suggesting that *MdTFL1* affects the flower development as well as flower induction in apple.

7. The future use of the techniques to reduce the juvenile phase by regulating the expression of the *TFL1*-like genes should be of advantage in breeding, crop production, and basic research such as molecular studies on woody plants, including fruit trees.

Fig. 6-1 Comparison of amino acid sequences of MdAP1 with those of apple and *Arabidopsis MADS*-box genes

Dashed circle represents *AP1/CAL* group.
AP1, APETALA1; CAL, CAULIFLOWER; FUL, FRUITFULL.

Fig. 6-2 Breeding strategy using precocious flowering lines

Fig. 6-3. Co-expression and re-introduction systems for early analysis of genes expressed in fruits (A) and transformation vector used in co-expression system (B)

MCS, multi cloning site, P, promoter; T, terminator.

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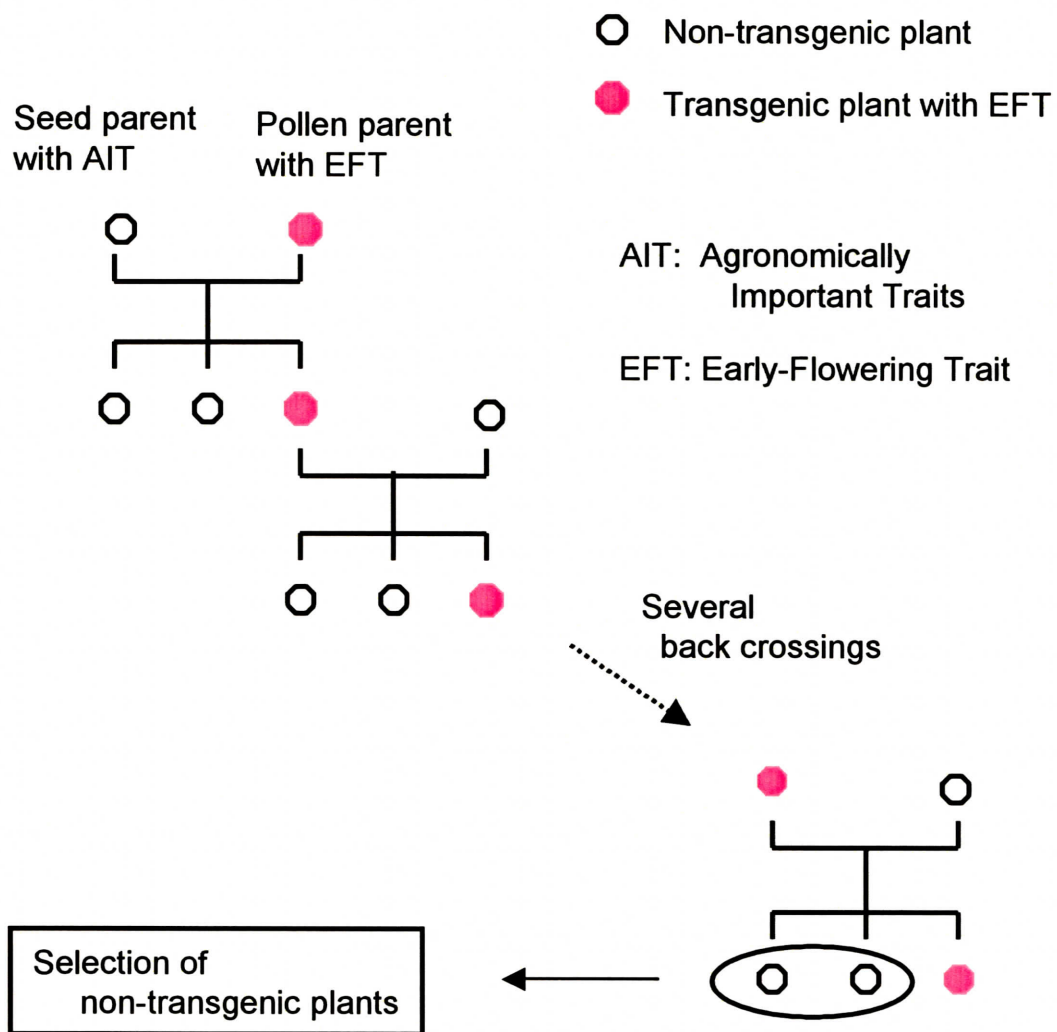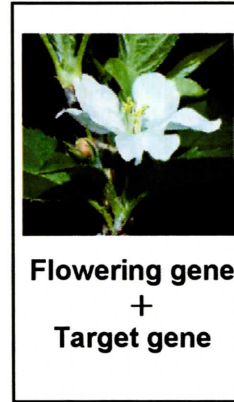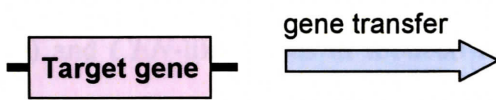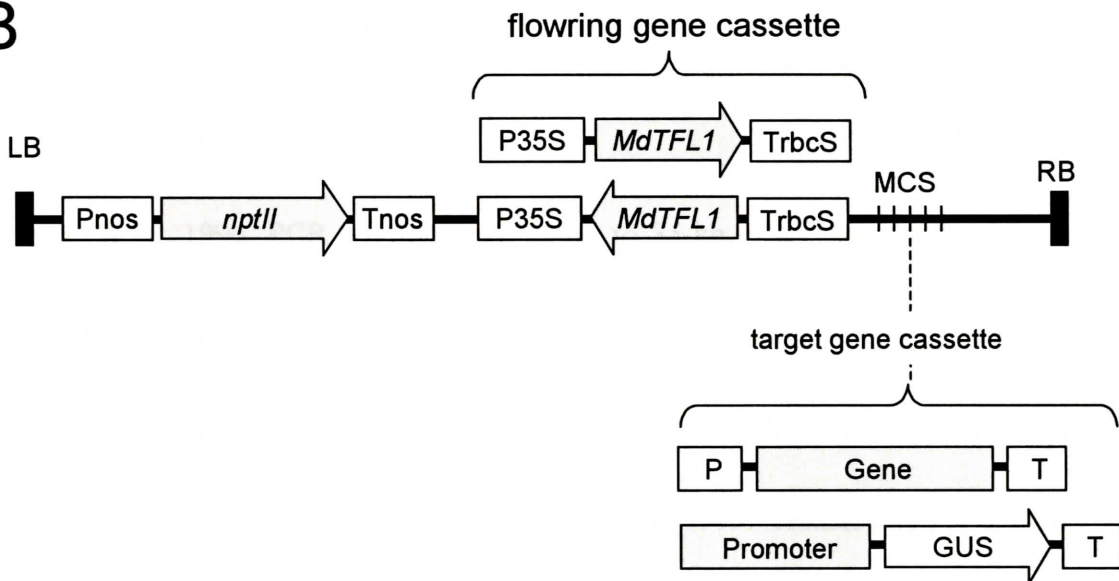Amaya, I., O.J. Ratcliffe, and D.J. Bradley. 1999. Expression of *CENTRORADIALIS* (*CEN*) and *CEN*-like genes in tobacco reveals a conserved mechanism controlling phase change in diverse species. Plant Cell 11:1405-1417.

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