ELSEVIER

Plant Science 162 (2002) 679–687

PLANT SCIENCE

www.elsevier.com/locate/plantsci

Overexpression of *MdMADS5*, an *APETALA1*-like gene of apple, causes early flowering in transgenic *Arabidopsis*

Nobuhiro Kotoda [a,*], Masato Wada [a], Shinnosuke Kusaba [b], Yuriko Kano-Murakami [b], Tetsuo Masuda [a], Junichi Soejima [a]

[a] *Department of Apple Research, National Institute of Fruit Tree Science, National Agricultural Research Organization, Shimo-kuriyagawa, Morioka 020-0123, Japan*

[b] *National Institute of Fruit Tree Science, National Agricultural Research Organization, Tsukuba 305-8605, Japan*

Received 10 August 2001; received in revised form 28 December 2001; accepted 2 January 2002

Abstract

An apple (*Malus* × *domestica*) gene *MdMADS5*, a putative homolog of *Arabidopsis APETALA1* (*AP1*) and *Antirrhinum SQUAMOSA*, 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_1) plants flowered 5–10 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.

Keywords: *APETALA1*; *Arabidopsis thaliana*; Early flowering; MADS-box; *Malus* × *domestica*

1. 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* [1–3]. Genes that control flowering time have been isolated and they include *LEAFY* (*LFY*) [4], *APETALA1* (*AP1*) [5], *CAULIFLOWER* [6], and *TERMINAL FLOWER 1* (*TFL1*) [7–9] in *Arabidopsis*. Overexpression of *LFY* [10] and *AP1* [11] shortened the juvenile period and caused early flowering, whereas overexpression of *TFL1* delayed flowering [12].

Genetic studies on morphogenesis in apple have started now that these genes related to plant development, including flower initiation, have been isolated in *Arabidopsis*. In apple, *MdMADS1* [13] which has a MADS-box domain [14] was isolated based on work in *Arabidopsis*. As for genes homologous to *AP1*, *MdMADS2* [15] and *MdMADS5* [16,17] were cloned and characterized. Overexpression of *MdMADS2* was found to cause early flowering in tobacco (*Nicotiana tabacum*) [15]. In our previous study, we cloned a fragment of *MdMADS5* and analyzed it to determine the relationship between floral bud formation and its expression. In apple, *MdMADS5* was expressed in sepals 2 months after flower-bud differentiation [17]. Recently, Yao et al. [18] cloned the apple *MdPI* homologous to *PISTILLATA* (PI) [19] and identified that *MdPI* has been mutated by a retrotransposon insertion in apetalous mutants such as 'Rae Ime' and 'Spencer Seedless'. Although several apple genes including MADS-box genes have been isolated, few of them

* Corresponding author. Tel.: +81-19-641-3164; fax: +81-19-641-3819.

E-mail address: koto@affrc.go.jp (N. Kotoda).

0168-9452/02/$ - see front matter
PII: S0168-9452(02)00024-9

are focused on flower induction and how they function in plants.

In this paper, we describe the effect of the apple *MdMADS5* gene, a putative homolog of *AP1*, on flowering time in transgenic *Arabidopsis* and discuss the possibility of involvement of *MdMADS5* on flower development in apple.

2. Materials and methods

2.1. Plant materials

The apple (*Malus* × *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.

2.2. Extraction of DNA from apple

DNA was isolated from the 'Jonathan' apple by cetyltrimethylammonium bromide (CTAB)-based methods [20]. Approximately 3 g of young leaves 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 centrifuged at 12 000g_n for 5 min at 25 °C in a swing bucket rotor KS-5000P (Kubota Co., Tokyo, Japan). The pellet was then resuspended in 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. 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 loop, then redissolved in 5 ml of 1 M NaCl containing RNase at a concentration of 10 mg ml^{-1} in a 15 ml tube, and incubated at 56 °C for 2–3 h. 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 TE (10 mM Tris–HCl pH 8.0, and 1 mM EDTA) and stored at 4 °C.

2.3. DNA blotting analysis

Ten micrograms of genomic DNA was digested with restriction enzymes, *Bam*HI, *Eco*RI, and *Hin*dIII and electorophoresed on 0.8% agarose gel (FMC Bioproducts, Rockland, ME, 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 amplified between squa/ap1-1S and -5A primers was used as a template of PCR probes for DNA gel blot analysis (Fig. 1A and Table 1). Hybridization was performed at 65 °C 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 h.

2.4. Gene cloning of MdMADS5 and vector construction for the expression of the MdMADS5 gene

Since *MdMADS5* was expressed exclusively in sepals [17], an apple cDNA library from sepals was used as template to clone a full length *MdMADS5* cDNA by TaKaRa LA PCR^{TM} in vitro cloning kit (Takara Biomedicals, Tokyo, Japan). After the cDNA synthesis, an *Eco*RI cassette was ligated to the cDNA. Then, 5′ rapid amplification of cDNA ends (RACE)- and 3′ RACE-PCR were performed using a set of adaptor primers and two antisense primers, and a set of adaptor primers and two sense primers, respectively (Fig. 1A and Table 1). Because the fragment of *AP1*-like gene we cloned was found to be identical to *MdMADS5* [16], the corresponding 1.0-kb cDNA was amplified by using long and accurate (LA)-PCR [21]. Specific sense primer 1S, derived from information of gene bank data registered by Yao et al. [16] and a specific antisense primer 7A, derived from a 600-bp fragment amplified by 3′ RACE-PCR were used (Table 1). 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).

The pUC119 plasmid vector, pUMDAP1.2+, containing *MdMADS5* cDNA, was digested with *Xba*I and *Sac*I, ligated to the binary vector pSMAK 251 [22] in a sense-oriented manner under the CaMV 35S promoter, resulting in pSMDAP1.1+ (Fig. 1B). It was then introduced into a disarmed strain of *Agrobacterium tumefaciens*, EHA101 [23]. Selectable marker genes encode kanamycin resistance for plants and spectinomycin for bacteria.

2.5. Arabidopsis transformation

An *A. tumefaciens* strain EHA101 was used to transform *Arabidopsis* Columbia plants by a floral-dip method [24]. To select transformed plants, the sterilized seeds were suspended in 0.1% sterile agarose, plated on

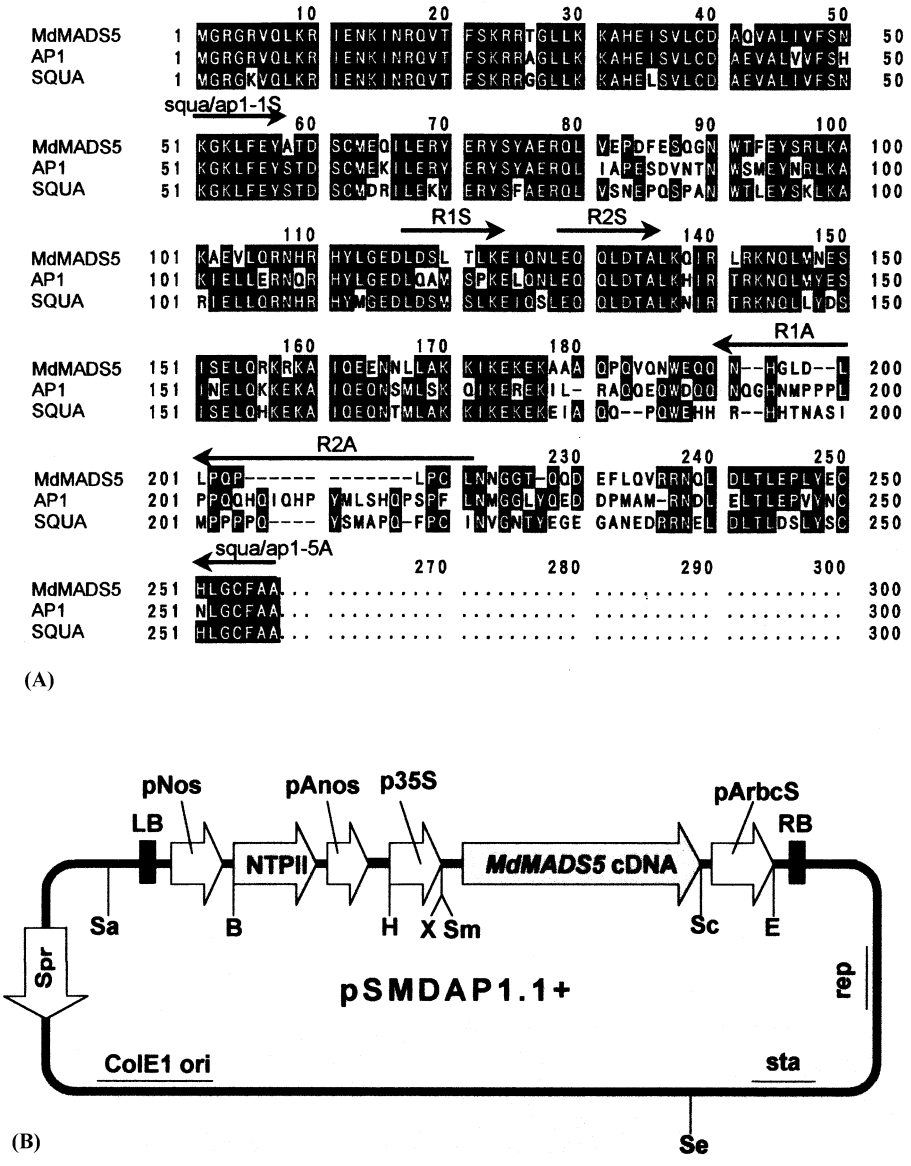

Fig. 1. Sequence comparison of *MdMADS5* and vector construction. (A) Comparison of the *MdMADS5* sequence with its homolog *AP1* [5] in *Arabidopsis* and *SQUA* [29] in *Antirrhinum*. Amino acids at positions of identity between two or three of these proteins are blocked in black. Arrows above sequences indicate primer sites. (B) 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 *rbcS*; 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.

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 h light/8 h dark). The selection plates contained one half strength of 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_1) and subsequent generations.

2.6. RNA analysis by RT-PCR

Detection of *MdMADS5* transcripts by RT-PCR was performed by using the RT-PCR kit (Toyobo, Tokyo, Japan). As an internal control, the actin *AAc1* gene [25]

Table 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′-GTACATATTGTCGT-TAGAACGCGTAATAC-GACTCA-3′
C2 primer	5′-CGTTAGACGCGTA-ATACGACTCACTATA-GGAGA-3′
5′ RACE primers	
R1A antisense primer	5′-AAGCAGGTCAAGG-CCATGGTTCTG-3′
R2A antisense primer	5′-TTAGACACATGGAA-GTGGCTGTGG-3′
3′ RACE primers	
R1S sense primer	5′-CCTGGATTCCTTGA-CTCTCAAGG-3′
R2S sense primer	5′-TTTGGAGCAACAG-CTTGACACCG-3′
MdMADS5 specific primers	
1S sense primer	5′-CAGTTTCTGGGTT-GTCTTTC-3′
7A antisense primer	5′-ACTCAATATTTCT-CAGGTTT-3′

was employed. RNA was isolated from whole plants of *Arabidopsis* by a method using CTAB described previously [17]. *MdMADS5*- and *AAc1*-specific transcripts were identified using 1 μg of total RNA as a template and the following primers: a sense primer 1S and an antisense primer 7A for *MdMADS5* (Table 1), 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 Aacl, giving rise to a 1037- and 457-bp long PCR product, respectively. PCR reactions were run for 40 cycles at 50 °C for *MdMADS5* and at 58 °C for *AAc1*. The PCR products were run on 1.5% (w/v) agarose gels stained with ethidium bromide.

3. Results

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 there might be a gene similar to *MdMADS5* (Fig. 2).

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 2). Five of the 15 primarily transformed plants (T_1 generation) carrying 35S::*MdMADS5* flowered earlier than the wild-type plants by 5–10 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 resemble those of *tfl1* mutant plants [9]. Similar results were obtained in T_1 transgenic *Arabidopsis* with 35S::*AP1* (data not shown). This phenotype observed in T_1 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_2 Transgenic line No. 12-1 showed early flowering although no differences in appearance were seen between T_1 transgenic line No. 12 and the wild-type plants. Quantitative characteristics of five independent transgenic lines (T_3 generation) are shown in Table 3. Typical 35S::*MdMADS5* transformants (T_4 seedlings from the transgenic line No. 3-1-7) that flowered 17 days after sowing are shown in Fig. 3A and B. The wild-type plant had eight rosette leaves and no flower buds on day 20 (Fig. 3C). The inflorescence of transformants with early flowering was shorter (< 3 cm) and numbers of rosette leaves were extremely reduced (2–5) at flowering as compared to controls (Fig. 3B and Table 3). In wild-type *Arabidopsis*, flower consists of four floral organs (four sepals, four petals, six stamens,

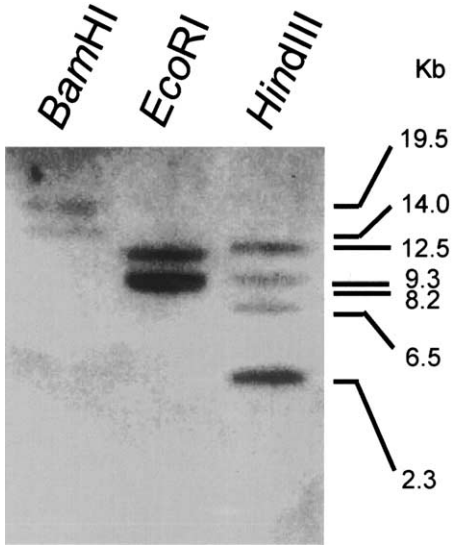

Fig. 2. DNA blot analysis. Equal amounts (10 μg) of apple DNA were blotted onto a Hybond N^+ filter. Restriction enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII, from left to right) used to digest apple genomic DNA are indicated at the top. Hybridization was done with a DIG-labeled PCR probe lacking the MADS-box region of *MdMADS5*. The numbers to the right represent the size of the DNA in Kb.

Table 2
Comparison of flowering time, number of rosette leaves in T_1 transgenic and wild-type *Arabidopsis* (Col) plants in LD conditions

Transgenic line	Days to flowering[a]	Rosette leaves at time of flowering[b]	Note
1	18	3	Early flowering
2	16	2	Early flowering
3	16	3	Early flowering
4	20	2	Early flowering
5	20	2	Early flowering
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[c]	25.5[d]	9.5[d]	($n = 10$)[e]

[a] Days to flowering is defined as the time when flower primordia were first visible to the naked eye.
[b] Rosette leaves were counted on the day that flower primordia were first visible.
[c] wt = wild-type *Arabidopsis* columbia plants.
[d] Mean number.
[e] Number of plants.

and a pistil) (Fig. 3D, E). However, in transgenic lines with a severe phenotype, abnormal floral organs were observed (Fig. 3F, G) and some were less fertile.

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_3 transgenic *Arabidopsis* plants were grown under both LD (16 h light/8 h dark) and SD (8 h light/16 h dark) photoperiods (Table 3). Ten to 15 seeds from each T_2 transgenic line (Nos. 2-3, 3-1, 4-1, 5-3, and 12-1) derived from T_1 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 9 weeks to flower.

The expression of the *MdMADS5* mRNA in T_1 plants was confirmed by RT-PCR (Fig. 4). 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. 4, wt). The expression of *MdMADS5* mRNA was detected in all of the kanamycin-resistant plants tested for expression analysis. The *MdMADS5* mRNA was expressed relatively weakly in five lines that flowered early (Fig. 4, lanes 1–5), compared to other lines. It was detected only weakly in the line No. 5 plant, although the line No. 5 plant flowered earlier and produced far fewer rosette leaves than the wild-type plants.

4. 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 and Table 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 2–3 months after floral-bud differentia-

Table 3
Comparison of flowering time, number of rosette leaves in T_3 transgenic and wild-type *Arabidopsis* (Col) plants grown in LD and SD conditions

Line	LD conditions (16 h light/8 h dark)				SD conditions (8 h light/16 h dark)			
	Days to flowering[a]	Rosette leaves at time of flowering[b]	Length of inflorescence (cm)[c]	Number of plants	Days to flowering[a]	Rosette leaves at time of flowering[b]	Length of inflorescence (cm)[c]	Number 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

[a] Days to flowering is defined as the time when flower primordia were first visible to the naked eye. Values are mean±SEM.
[b] Rosette leaves were counted on the day that flower primordia were first visible. Values are mean±SEM.
[c] Length of inflorescence was measured a month after sowing. Values are mean±SEM.

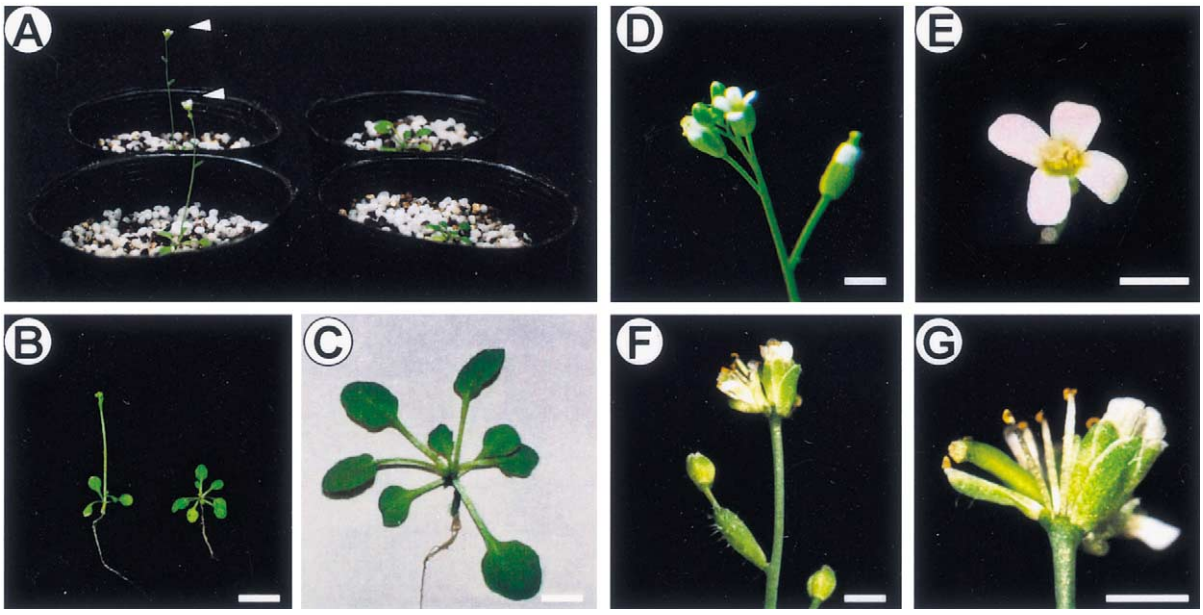

Fig. 3. Transgenic and wild-type *Arabidopsis* plants grown under LD photoperiods for 20 days. *Arabidopsis* plants were grown in one-half strength of MS medium for 10 days and transferred to potting soil. (A) Transgenic *Arabidopsis* carrying a 35S::*MdMADS5* gene (left) and wild-type *Arabidopsis* 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. Scale bar = 10 mm (B), 3 mm (C) and 2 mm (D, E, F, G). Photos were taken 20 (A, B, C, F, G) and 30 days (D, E) after sowing.

tion concurrent with sepal formation of apple [17]. 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 [26]. Thus, one of factors that limits breeding strategies in tree fruits is the long juvenile phase that lasts several years [27]. Several years ago, it was found that transgenic approaches could reduce the juvenile phase of *Populus* by introducing

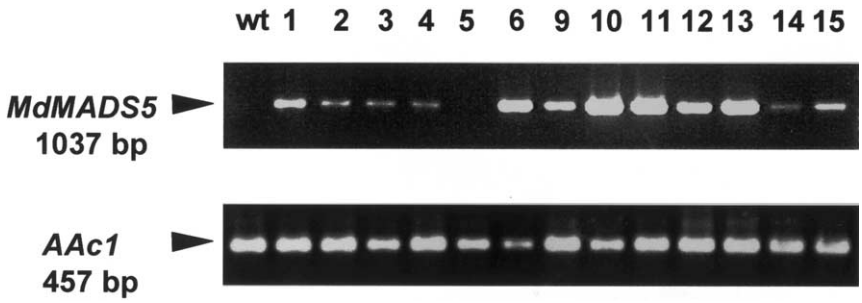

Fig. 4. Analysis of *MdMADS5* expression in transgenic *Arabidopsis*. Detection of *MdMADS5* was performed by RT-PCR. *MdMADS5* was identified using 1 μg of the total RNA as a template and the specific primers, giving rise to a PCR product of 1037 bp. PCR products were run on 1.5% (w/v) agarose gel stained 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.

LFY under the CaMV 35S promoter and the transgenic poplar flowered 5 months after regeneration [10]. 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., unpublished results). Recently, Peña et al. [28] 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.

Acknowledgements

We gratefully acknowledge Dr H. Ichikawa, National Institute of Agrobiological Resources, Tsukuba, Japan, and Professor M. Yanofsky, the University of California, San Diego, for providing us a binary vector pSMAK251 and pAM563 (35S::*AP1* construct), respectively. We also thank Dr C. Honda, National Institute of Fruit Tree Science, Tsukuba, Japan, for technical advice. This research was supported by a grant from the Ministry of Agriculture, Forestry, and Fisheries (MAFF) for the 'development of innovative plants and animals using transformation and cloning'. Contribution No. 1244 of National Institute of Fruit Tree Science.

References

[1] Y.Y. Levy, C. Dean, The transition to flowering, Plant Cell 10 (1998) 1973–1989.
[2] M.S. Pidokowich, J.E. Klenz, G.W. Haughn, The making of flower: control of floral meristem identity in *Arabidopsis*, Trends Plant Sci. 4 (1999) 64–70.
[3] M. Piñeiro, G. Coupland, The control of flowering time and floral identity in *Arabidopsis*, Plant Physiol. 117 (1998) 1–8.
[4] D. Weigel, J. Alvarez, D.R. Smyth, M.F. Yanofsky, E.M. Meyerowitz, *LEAFY* controls floral meristem identity in *Arabidopsis*, Cell 69 (1992) 843–859.
[5] M.A. Mandel, C. Gustafson-Brown, B. Savidge, M.F. Yanofsky, Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*, Nature 360 (1992) 273–277.
[6] S.A. Kempin, B. Savidge, M.F. Yanofsky, Molecular basis of the *cauliflower* phenotype in *Arabidopsis*, Science 267 (1995) 522–525.
[7] D. Bradley, O. Ratcliffe, C. Vincent, R. Carpenter, E. Coen, Inflorescence commitment and architecture in *Arabidopsis*, Science 275 (1997) 80–83.
[8] S. Ohshima, M. Murata, W. Sakamoto, Y. Ogura, F. Motoyoshi, Cloning and molecular analysis of the *Arabidopsis* gene *Terminal Flower 1*, Mol. Gen. Genet. 254 (1997) 186–194.
[9] S. Shannon, D.R. Meeks-Wagner, A mutation in the arabidopsis *TFL1* gene affects inflorescence meristem development, Plant Cell 3 (1991) 877–892.
[10] D. Weigel, O. Nilsson, A developmental switch sufficient for flower initiation in diverse plants, Nature 377 (1995) 495–500.
[11] M.A. Mandel, M.F. Yanofsky, A gene triggering flower formation in *Arabidopsis*, Nature 377 (1995) 522–524.
[12] O.J. Ratcliffe, I. Amaya, C.A. Vincent, S. Rothstein, R. Carpenter, E.S. Coen, D.J. Bradley, A common mechanism controls the life cycle and architecture of plants, Development 125 (1998) 1609–1615.
[13] S.-K. Sung, G. An, Molecular cloning and characterization of a MADS-box cDNA clone of the Fuji apple, Plant Cell Physiol. 38 (1997) 484–489.
[14] J.L. Riechmann, E.M. Meyerowitz, MADS domain proteins in plant development, Biol. Chem. 378 (1997) 1079–1101.
[15] S.-K. Sung, G.-H. Yu, G. An, Characterization of *MdMADS2*, a member of the *SQUAMOSA* subfamily of genes, in apple, Plant Physiol. 120 (1999) 969–978.
[16] J.-L. Yao, Y.-H. Dong, A. Kvarnheden, B. Morris, Seven MADS-box genes in apple are expressed in different parts of the fruit, J. Am. Soc. Hortic. Sci. 124 (1999) 8–13.
[17] N. Kotoda, M. Wada, S. Komori, S. Kidou, K. Abe, T. Masuda, J. Soejima, Expression pattern of homologues of floral meristem identity genes *LFY* and *AP1* during flower development in apple, J. Am. Soc. Hortic. Sci. 125 (2000) 398–403.
[18] J.-L. Yao, Y.-H. Dong, B. Morris, Parthenocarpic apple fruit production conferred by transposon insertion mutations in a MADS-box transcription factor, Proc. Natl. Acad. Sci. USA 98 (2001) 1306–1311.
[19] K. Goto, E.M. Meyerowitz, Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*, Genes Dev. 8 (1994) 1548–1560.
[20] G.C. Murray, W.F. Thompson, Rapid isolation of high molecular weight DNA, Nucleic Acid Res. 8 (1980) 4321–4325.
[21] W.M. Barnes, PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates, Proc. Natl. Acad. Sci. USA 91 (1994) 2216–2220.
[22] S. Yamashita, H. Ichikawa, Y. Ito, Y. Ohashi, Development of new binary vectors stably maintained in *Agrobacterium* and their utilization to plant transformation, Breeding Sci. 45 (suppl. 1) (1995) 56.
[23] E.E. Hood, G.L. Helmer, R.T. Fraley, M.-D. Chilton, The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA, J. Bacteriol. 168 (1986) 1291–1301.
[24] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.
[25] C.J. Nairn, L. Winesett, R.J. Ferl, Nucleotide sequence of an actin gene from *Arabidopsis thaliana*, Gene 65 (1988) 247–257.
[26] S. Sadamori, Y. Yoshida, H. Murakami, S. Ishizuka, New apple variety 'Fuji', Bull. Hortic. Res. Sta. Jpn. Ser. (C) 1 (1963) 1–6.
[27] W.P. Hackett, Juvenility, maturation, and rejuvenility in woody plants, Hortic. Rev. 7 (1985) 109–155.
[28] L. Peña, M. Martín-Trillo, J. Juáres, J.A. Pina, L. Navarro, J.M. Martinez-Zapater, Constitutive expression of *Arabidopsis*

LEAFY or *AP1* genes in citrus reduces their generation time, Nat. Biotechnol. 19 (2001) 263–267.
[29] P. Huijser, J. Klein, W.-E. Lönnig, H. Meijer, H. Sommer, Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene *squamosa* in *Antirrhinum majus*, EMBO J. 11 (1992) 1239–1249.