The Break-through in the Reduction of Juvenile Phase in Apple Using Transgenic Approaches

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Abstract

In contrast to herbaceous plants, fruit trees such as apple [*Malus* x *domestica* (Borkh.)] flower and set fruit only after an extended juvenile phase that lasts several years. In the course of studying juvenility in apple, we have cloned the *MdTFL* gene homologous to *Arabidopsis TERMINAL FLOWER1* (*TFL1*) gene, which suppresses floral meristem identity genes, *LEAFY* (*LFY*) and *APETALA1* (*AP1*). The expression of *MdTFL* (*Malus* x *domestica TFL1*) mRNA was strong in sepals and mature leaves, and highest in early July, about two weeks before flower induction, in apical buds in apple. Transgenic apples expressing antisense *MdTFL* gene flowered at about eight to 15 months after grafting onto rootstocks. On the other hand, non-transformed control plants have not flowered in five years. The flower organs of the transgenic apples were normal and fertile. These results demonstrate that the suppression of the expression of *MdTFL* gene reduces the juvenile phase and can induce precocious flowers in apple.

INTRODUCTION

In the development of all woody plants from seed, there is a so-called juvenile phase lasting up to 30-40 years in certain forest trees, during which flowering does not occur and can not be induced by normal conditions (Hackett, 1985). Therefore, the breeding of fruit trees such as apple (*Malus* x *domestica*) often requires more than 20 years, including the 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, set the first fruits 12 years after sowing, and took 23 years before it was released as a variety (Sadamori et al., 1963). Generally, the juvenile period in apple (*Malus* x *domestica*) can last four to eight years, but a certain *Malus* sp. used as root stock neither flowers nor sets fruits for many years. Thus, the greatest factor that limits breeding efficiency of fruit trees is their long juvenile phase. It is important to understand the mechanism of flower induction and development, and apply it to the improvement and production of the fruit, because apple is one of the most commercially important tree fruits in the world. However, the genetic factors controlling flower induction in apples have not been investigated in detail. To investigate the possible factor responsible for the maintenance of juvenility in apple, we have cloned the *AFL*, *MdAP1*(MdMADS5), and *MdTFL* genes, putative apple homologs of *LFY* (Weigel et al., 1992, 1995), *AP1* (Mandel et al., 1992, 1995), and *TFL1* (Bradley et al., 1997; Oshima et al., 1997), respectively (Kotoda et al., 2000, 2002; Wada et al., 2002; Yao et al., 1999). In addition, we produced transgenic apples expressing sense or antisense of these genes for early flowering. In this paper, we describe isolation and characterization of *MdTFL*, and precocious flowering of apple by expressing antisense *MdTFL*.

MATERIALS AND METHODS

Plant Materials

The apple [*Malus* x *domestica* (Borkh.)] cultivar, Jonathan, was used in this study to isolate and characterize the *MdTFL* gene. The apple cultivar Orin, and *Arabidopsis thaliana* (ecotype Columbia) were used for *Agrobacterium*-mediated transformation.

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Apple leaves and flowers were collected from the experimental field or greenhouses at our research center in Morioka, Japan.

Construction of Transformation Vectors

The plasmid vector pUMDTFL12.1+ containing *MdTFL* cDNA was cut with *Xba*1 and *Sac*I, and the *MdTFL* cDNA was ligated to a binary vector pSMAK251 (Yamashita et al., 1995), cut with the same restriction enzymes, in a sense oriented manner to yield pSMDTFL12.1.2+. The plasmid vector pUMDTFL1- was cut with *Xba*1 and *Sac*I, and ligated to a binary vector pSMAK251 in an antisense oriented manner to yield pSMDTFL1.1-.

Transformation of Apple

An *Agrobacterium* strain EHA101 containing pSMDTFL1.1- was used for transformation of apple leaves. The *MdTFL* genes were controlled by the CaMV 35S promoter. *Agrobacterium* was grown overnight and induced by acetosyringone. Apple leaves were inoculated with 0.5 OD inoculum for 30 min. The leaf explants were transferred to a co-cultivation medium and incubated in the dark. After 1 week, the cultures were transferred to antibiotic selection plates containing Murashige and Skoog (MS) medium, 25 mg L^{-1} kanamycin, and 500 mg L^{-1} claforan. When adventitious shoots appeared, cultures were transferred to antibiotic selection plates containing MS, 50 mg L^{-1} kanamycin, and 500 mg L^{-1} claforan and incubated in the light. When shoots had produced several leaves, they were transferred to proliferation medium. After checking the transgene insertion by PCR using specific primers for *MdTFL*, shoots were transferred to MS medium free from antibiotics.

RNA Blot Analysis

RNA was isolated from 'Jonathan' apple at various developmental stages. RNA was separated on a 1.2% agarose gel containing 5.0% (v/v) formaldehyde. Total RNA (20 µg) was loaded per lane. The gels were blotted onto Hybond-N+ (Amersham). Hybridization was done with DIG-labelled sense and antisense RNA probes, which were synthesized from pBMDTFL12+ cut with *Eco*RI and *Sal*I by using T7 and T3 RNA polymerases, respectively. Hybridization and washing were performed according to a standard protocol (Boehringer Mannheim). The resulting images were analyzed by a LAS-1000 image analyzer (Fujifilm).

RESULTS

Gene Cloning and Expression Pattern of MdTFL

To investigate the genes possibly related to juvenility in apple, we have cloned the *MdTFL* gene, a putative homologue of *Arabidopsis TFL1*, by RACE methods. The primers used in cloning are shown in Fig. 1A. The predicted protein of the *MdTFL* with 172 amino-acid residues had a greater similarity to *TFL1* rather than to *CEN*: 74% amino-acid residues were shared with *TFL1*, 72% with *CEN* (Bradley et al., 1996), 72% with *SP* (Pnueli et al., 1998), and 56% with *FLOWERING LOCUS T* (*FT*) (Kardilsky et al., 1999; Kobayashi et al., 1999) which acts in an antagonistic manner to *TFL1*. Therefore, this gene was designated *MdTFL* after *Malus* x *domestica TFL1* homolog. DNA blotting was performed using apple DNA digested with *Bam*HI, *Eco*RI, *Hind*III, *Nco*I, *Xba*I, and *Xho*I probed with *MdTFL*. Two to four bands were detected in DNA blotting analysis, suggesting that there is an unknown gene homologous to *MdTFL*. In fact, it is reported that *Arabidopsis* genome contains six *TFL1*-like genes, forming a gene family (Mimida et al., 2001). The coding region of *MdTFL* cDNA was used to analyze the expression of *MdTFL* both in various living tissues and in the apices of current shoots in apple at different developmental stages by generating an antisense digoxigenin (DIG)-labeled RNA probe. The *MdTFL* mRNA was expressed preferentially in sepals and mature leaves, but very weakly in petals, apical buds and cotyledons of seedlings. There was no expression in

stamens, carpels, stems and roots. *MdTFL* mRNA from the apices of apple was looked at throughout the season. *MdTFL* mRNA was expressed strongly in early July, about two weeks before the occurrence of flower induction in apple. It decreased gradually from July to mid-December, with an increase to April concurrent with sepal and leaf development. Interestingly, the seasonal expression pattern of *MdTFL* in the apices of apple appeared to be opposite to that of *AFL* gene (Kotoda et al., 2000).

Transgenic Arabidopsis with MdTFL

To determine whether *MdTFL* gene functions as *TFL1*, we constructed a binary vector pSMDTFL12.1.2+ or pSMDTFL1.1- where full-length *MdTFL* cDNA was inserted in sense or antisense oriented manner, respectively, under the control of 35S CaMV promoter. The vector carrying *MdTFL* cDNA (pSMDTFL12.1.2+) was introduced into wild type *Arabidopsis* plants (Columbia). Thirty-four independent transgenic plants that survived on kanamycin were identified. Eight of thirty-four primary transformants (T_1 generation) flowered later than wild-type plants and showed the similar phenotype to that of transgenic *Arabidopsis* overexpressing *TFL1*(Ratcliffe et al., 1998). The phenotype observed in the T_1 generation was inherited in the following generations. Quantitative characteristics of six independent T_2 transgenic lines are shown in Table 1. In the T_2 plants, the first flower opened 39.4 days after sowing and the latest flower opened at day 59, compared to 30.3 for the control plants (Table 1). Rosette leaf number, a measure of developmental time to flowering, was higher in transgenic plants and was as many as 23.0 for the seedlings from T-S22 as compared to 8.0 for the controls. The transformants had fifteen to twenty rosette leaves and neither flower buds nor bolting were seen, whereas the control plant had many flowers and brown pods at day 35. In addition, there was little difference between *Arabidopsis* plants transformed with a binary vector pSMDTFL1.1- carrying *MdTFL* antisense gene and control wild-type plants in flowering time and rosette numbers at flowering (data not shown). These results suggest that *MdTFL* functions like *TFL1* and it is an apple ortholog of *TFL1*.

Expression of MdTFL in Transgenic Apples

Based on these results described above, *MdTFL* was thought to function as the developmental switch between the vegetative and reproductive phase in apple like *TFL1* in *Arabidopsis*. If this is the case, there is a possibility that the suppression of the *MdTFL* gene would trigger flower induction during the vegetative phase of apple. To investigate this speculation, we produced transgenic apples overexpressing the *MdTFL* antisense gene. About two thousand leaf discs of apple variety ‘Orin’ were used for *Agrobacterium*-mediated transformation. After regeneration of transformed shoots from three independent leaf discs, the shoots were cultured for a month and the insertion of transgenes was confirmed by PCR using specific primers for nptII and *MdTFL* genes. The transformed shoots from each line were grafted onto rootstocks to produce 11 transgenic lines (Table 2), and these were transferred to an isolated greenhouse. In lines 303-1, 2, 3, and 4, the number of branches was relatively low and the branches were upright as compared to the other lines (Table 2). The lines 705-3 and 4 showed a weak growth habit. Under in vitro culture conditions, no flowers were produced in transgenic or control plants. The expression of the transgene was analyzed in leaves of transformants by RNA blot hybridization. The *MdTFL* antisense mRNA was detected by hybridizing with sense RNA probe, confirming that all the transgenic lines tested express *MdTFL* antisense mRNA in their leaves (Table 2). Especially, it was expressed strongly in transgenic lines 303-1, 303-4, and 705-1, although the expression level was relatively low in lines 705-3 and 705-4. To examine the effect of *MdTFL* antisense gene expression on the other apple genes, expression analysis of *AFL* and *MdMADS5* was performed using reverse transcription (RT) – PCR. Unexpectedly, in leaves, no difference in the gene expression was observed between transgenic and non-transgenic plants (data not shown).

Precocious Flowering in Transgenic Apples

Although apples flower naturally after four to eight years, the transgenic line 705-1 produced a solitary flower only eight months after grafting onto a rootstock. Normally, apple trees require chilling for several months before developing leaves and flowers in spring, but this line flowered without undergoing a dormancy period. On the other hand, such an effect was not observed in non-transgenic controls. In addition, not a single instance of precocious flowers has been seen in more than 100 other lines that were produced with various constructs during the past 6 years at the National Institute of Fruit Tree Science. The first flower of the line 705-1 was normal in appearance except that the stamens were much shorter than those of non-transgenic plants. After the break of the first dormancy, six transgenic lines, including the line 705-1, produced solitary flowers in the axils of leaves and at the top of two-year-old shoots or at the top of current shoots. But normal apple trees produce many clusters which consist of about five flowers on fruit bearing shoots (not on current shoots). In transgenic lines, some flowers appeared normal and others showed homeotic conversions resulting in flowers with more petals or without pistils. In the morphology of leaves, 303 lines showed round-shaped and more serrate leaves and 705-1 and 705-2 lines had leaves with much smaller stipules than those of controls.

Line 705-4 started to flower one month later than line 705-1. Surprisingly, it has been producing flowers continuously for at least three months. In nature, flower induction of apple occurs about two month after anthesis, and flower organ primordia are formed during the period from summer to the next spring (Kotoda et al., 2000). In general, apple trees neither produce flowers nor set fruits on any current shoots under normal conditions. However, it is likely that flower induction in line 705-4 occurs at any time and the period required for the development of flower organs is reduced extremely. Such a phenomenon was also observed in the transgenic line 705-3.

To confirm the fertility of the transgenic apples showing early flowering, we carried out crossing and pollen germination tests. Line 705-1 crossed with a Japanese apple cultivar ‘Sansa’ set three fruits out of five flowers. One of the young fruits is developing. Furthermore, pollen from line 705-1 was used to pollinate a columnar type apple ‘Wijcik’. The development of these transgenic fruits appeared to be a little slower than those of wild-type plants. Pollen from early flowering lines tested had the ability to germinate on a plate containing 1% agar and 17% sucrose, as did the controls (Table 2).

DISCUSSION

Environmental conditions that promote rapid, continuous growth generally reduce the length of the juvenile period. In apple, seedling scions grafted onto dwarfing rootstocks flower two to four years earlier than the seedlings from which they were taken (Hackett, 1985). There is a report that apple seedling progenies grown continuously in optimal greenhouse conditions flowered 16-20 months after germination by using defoliation to break dormancy but that seedlings from the same crosses grown in the field had not begun to flower four years after germination (Aldwinckle, 1975). From our experience, however, it would be difficult to reduce the juvenile period to less than two years in apple. In producing transgenic plants for early flowering, there are two ways of thinking. One is overexpressing the genes that promote flowering such as *LFY* and *AP1*, and second is suppressing the genes which delay flowering such as *TFL1*. We hypothesized that suppressing the late flowering genes could reduce the juvenile phase and induce precocious flowers more effectively than promoting the early flowering genes in woody plants, because only a few successes have been reported on precocious flowering in woody plants by overexpressing *LFY* or *AP1*. In this study, we showed that the expression of *MdTFL* antisense gene could induce precocious flowers in apple effectively. Although we produced several transgenic apples overexpressing genes possibly involved in the flowering switch including *FLO*, *AP1*, and their putative apple homolog genes *AFL* and *MdMADS5*, respectively, there have been no transgenic apples other than those expressing the *MdTFL* antisense gene that induces precocious flowering. In contrast with apple, citrus

overexpressing *LFY* or *AP1* produced precocious flowers 12-20 months after their transfer to the greenhouse (Peña et al., 2001). In apple, it is likely that *MdTFL* plays an important role rather than *AFL* or *MdMADS5* in regulating the transition to flowering. In woody plants, the maintenance of juvenile phase should be one of the most important events at the early stage of the plant development. However, it is interesting that the juvenile phase could be regulated by a small number of genes such as *TFL1*-like genes.

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Tables

Table 1. Phenotype of T_2 transformed *Arabidopsis* with *MdTFL* sense gene.

Transgenic line[a]	Days to flowering[b]	Rosettes at time of flowering[c]	No. of plants
Control	30.3 ± 1.6	8.0 ± 1.0	10
T-S6	39.4 ± 5.1	11.8 ± 3.6	5
T-S10	59.0 ± 15.6	15.6 ± 4.3	5
T-S11	45.4 ± 6.0	11.8 ± 2.3	5
T-S21	44.7 ± 6.9	18.0 ± 4.3	3
T-S22	47.0 ± 0	23.0 ± 4.6	3
T-S28	53.0 ± 6.0	21.5 ± 1.5	2

[a] Non-transgenic controls and seedlings from primary transformants (T_1) with MdTFL gene were grown under long-day (16hr light/8hr dark) conditions.
[b] Days to flowering is defined as the time when flower primordia were first visible to the naked eye.
[c] Rosettes were counted on the day that flower primordia were first visible.
[b,c] Values are means ± SEM.

Table 2. Phenotype of transformed apples with *MdTFL* antisense gene.

Transgenic line	transgene mRNA[a]	Time to flower (months)[b]	Growth habit[c]	Pollen viability	Grafting (yr/month)
Control[d]	ND	-	N	-	1997/8
303-1	+++	11	LB	NT	2000/6
303-2	++	-	LB	-	2000/6
303-3	++	-	LB	-	2000/6
303-4	+++	11	LB	NT	2000/6
705-1	+++	8	N	+	2000/4
705-2	++	15	N	+	2000/4
705-3	+	11	W, MB	+	2000/6
705-4	+	11	W, LB	+	2000/6
614-1	NT	-	N	-	2001/6
614-2	NT	-	N	-	2001/6
614-3	NT	-	N	-	2001/6

[a] mRNA was extracted from leaves and northern blot analysis was performed.
ND, not detected; NT, not tested.
[b] The months were counted after grafting.
[c] N, Normal growth habit; LB, less branched; MB, more branched; W, weak growth habit.
[d] Three independently regenerated non-transformed plants were used as controls.