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Apple has two orthologues of *FLORICAULA/LEAFY* involved in flowering

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Abstract

Two orthologues of *FLORICAULA/LEAFY, AFL1* and *AFL2* (apple *FLO/LFY*), were isolated from the floral buds of apple trees. Their expression was detected in various tissues and during differentiation of the floral buds. Furthermore, the flowering effectiveness of each gene was assessed with transgenic *Arabidopsis*. Both *AFL1* and *AFL2* showed high homology to each other (90%) and a high degree of similarity to *PTLF* and *PEAFLO* (70%), which are homologues of *FLO/LFY* from poplar and pea, respectively. RNA blot analysis showed that *AFL1* was expressed only in the floral bud during the transition from vegetative to reproductive growth, whereas *AFL2* was expressed in vegetative shoot apex, floral buds, floral organs and root. Genomic Southern analysis showed that apple had other homologues in addition to *AFL1* and *AFL2*. The transgenic *Arabidopsis* with over-expressed *AFL2* showed accelerated flowering and gave rise to several solitary flowers from rosette axils directly. *AFL1* had similar effects, but the phenotypes of the transgenic *Arabidopsis* with *AFL1* were weaker than those with *AFL2*. These results suggest that both genes are involved in flower differentiation in apple.

Introduction

Increasing knowledge of the flowering mechanism in *Arabidopsis* has opened the floodgate for the identification of several key genes that control floral development (Okamuro *et al.*, 1993; Levy and Dean 1998; Pidkowich *et al.*, 1999). One of such genes, *LEAFY (LFY)*, has been reported to be necessary for the transition from vegetative to reproductive development. It has been defined as a floral meristem identity gene like *FLORICAULA* (*FLO*) from *Antirrhinum majus* (Coen *et al.*, 1990). Mutations in these genes result in the conversion of flowers into indeterminate secondary shoots (Weigel *et al.*, 1992). The over-expression of *LFY* under the 35S promoter in *Arabidopsis* caused early flowering and converted all lateral shoots info solitary flowers. In heterologous plants such as aspen, *LFY* could have effects similar to acceleration of flowering and induction of ectopic flowers (Weigel and Nilsson 1995). These reports strongly suggest that the ability to control the expression of *LFY*, or of orthologues from other plants, could make it possible to induce various plants to blossom whenever and however possible. Consequently, these studies should be considered a contribution to agriculture and forestry.

Apple is one of the most commercially valuable fruit trees, with production second only to grape in the whole world. Apple has an extended juvenile phase, during which vegetative growth is maintained. This characteristic is recognized as a disadvantage in breeding and in stable annual production. Thus, it will be necessary to understand the genetic mechanism underlying transition from vegetative to reproductive phase. Only a few studies have reported on the mechanism underlying the development of apple flowers. Recently, MADS-box genes have been correlated to apple fruit set, and they have been cloned and characterized (Sung *et al.*, 1999, 2000). These apple MADS genes seem to be involved in floral organ and fruit

The nucleotide/amino acid sequence data reported will appear in the EMBL, GenBank and DDBJ databases under the accession numbers AB056158 (*AFL1*) and AB056159 (*AFL2*).

development rather than in the transition from the vegetative to the reproductive meristem. We have undertaken this study in apple to identify the homologues of *FLO/LFY* involved in floral development. In this paper, we describe the isolation and characterization of *LFY* homologues from apple floral apex during the vegetative-to-reproductive phase transition. In addition, the isolated genes were determined to have functional activity in *Arabidopsis* flowering.

Materials and methods

Plant materials

The apple (*Malus* × *domestica* Borkh.) cv. Jonathan, grown in our orchard at the Apple Research Center of the National Institute of Fruit Tree Science (Morioka, Japan), was used in this study. Developing floral shoot apexes 0.5–1.0 cm from the tip were cut and collected from early June to late August at two-week intervals. The cut apexes, including meristems, were frozen in liquid nitrogen immediately and stored at −80 °C.

RNA extraction and cDNA cloning

Total RNA was extracted from the cut apexes using a method based on cetyltrimethylammonium bromide (CTAB), and the cDNA was constructed by oligo(dT) primer with reverse transcriptase. Partial cDNA fragments homologous to *LFY* were amplified by RT-PCR (Kotoda *et al*., 2000). Full-length cDNA was obtained by using 5′ and 3′' RACE methods. The first amplified cDNA of apple was 440 bp between 6S (sense primer 5′-CAGAGGGAGCACCCGTTCATTGTGAC-3′) and 10A (antisense primer 5′-GACGC/AAGCTTT/GGTT/GGGA/GACATACCA-3′) designed from *Arabidopsis thaliana LFY* (Weigel *et al.*, 1992) and *Antirrhinum majus FLO* cDNA sequences (Coen *et al.*, 1990). This fragment was named *AFL400*. Specific primers for both 5′ and 3′ RACE methods were designed from this *AFL400*. The cassette-ligated cDNAs from the shoot apexes were prepared using the LA-PCR cloning kit (Takara, Tokyo). The amplification of the 3′ end of the cDNAs was carried out between cassette primer 1 or 2 and 6S or 3SP2 (5′-TCCAGAACATTGCCAAGGAG-3′) (see Figure 1). A 600 bp DNA fragment amplified C2-3SP2 was cloned at the *Eco*RV site using pBlueScript SKII+ (Stratagene, La Jolla, CA). Ten clones were completely sequenced in both directions and showed a division into two kinds of cDNA homologues. The amplification of the 5′ end was carried out between cassette primer 1 or 2 and 10A, 5SP2 (5′-CAATGACCACAAGAGGCTTG-3′), 5SP3 (complement sequence of 3SP2) or 5SP4 (complement sequence of 6S) (see Figure 1). An almost 750 bp band amplified C2-5SP4 was cloned and sequenced in the same way as in the 3′ RACE method. The resultant sequences also showed two kinds of cDNA. The full-length cDNAs were amplified by the shared sense primer *AFLF*, 5′-GTGGAAAATATGGATCCAGATGCC-3′, and either one of two antisense primers *AFL1R*, 5′-TTCATTCAGTCTGCCCTAGCC-3′, or *AFL2R*, 5′-TCAAACTCTCTCTGCAGAACTGGC-3′ (see Figure 1) from the cDNA library of floral shoot apexes. Fragments of about 1.4 kbp cDNA were obtained and subcloned into pBlueScript SKII+ (*Eco*RV site) and sequenced completely in both directions. Two kinds of cDNA were named *AFL1* and *AFL2*, respectively. The sequencing was performed by dideoxy methods using a Hitachi SQ5500S automated sequencer (Hitachi, Tokyo, Japan) and analyzed by the Genetyx computer analysis program (Software Development Co., Tokyo, Japan).

Southern blot and expression analyses

Genomic DNA was extracted by the method of Murray and colleagues (Murray and Thompson, 1980; Bousquet *et al.*, 1990). The Southern analysis was carried out using a 440 bp DIG-labeled *AFL400* PCR fragment amplified between 6S and 10A primers. Genomic DNA was digested by restriction enzyme *Eco*RI, *Bam*HI or *Hin*dIII, respectively, and blotted onto a nylon membrane (Hybond N+, Amersham). Hybridization was performed in DIG EASY HYB solution (Roche Biochemicals, Tokyo, Japan) overnight at 50 °C. The filter was rinsed twice in 2× SSC, 0.1% SDS at room temperature, and washed in 0.5× SSC, 0.1% SDS for 15 min, twice, at 65 °C. Chemiluminescence was detected according to the manufacturer's instructions, and the resulting images were analyzed with a LAS-1000 image analyzer (Fuji Film, Tokyo, Japan). RNA extraction from each tissue and organ was carried out by the same method as described above. First-strand cDNA synthesis was performed with 1.0 μg of total RNA using the RT-PCR HIGH kit (Toyobo, Tokyo, Japan) as directed by the manufacturer. The common sense primer was 3SP2 and the each antisense primer was *AFL1R* or *AFL2R*, the pair of 3SP2-*AFL1R* primers amplified a 532 bp fragment and the 3SP2-*AFL2R* primers ampli-

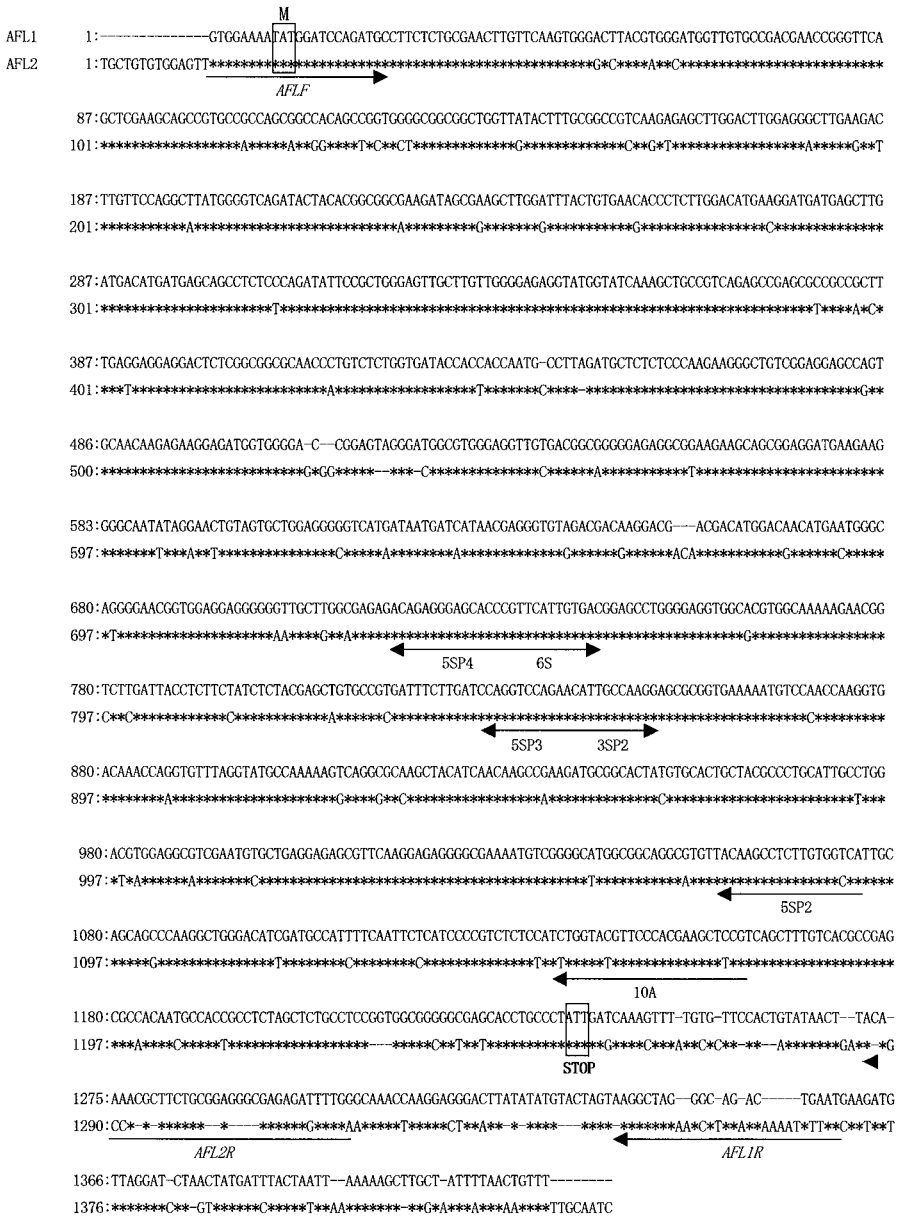

Figure 1. Alignment of nucleotide sequences of *AFL1* and *AFL2* cDNA. The upper line shows the nucleotide sequence of *AFL1* cDNA, and the lower line shows that of *AFL2* cDNA. Each primer used for RT-PCR or the RACE method is shown under each arrow. The 5SP4 and 6S primers, or 5SP3 and 3SP2, have the same position, but opposite directions; the arrowheads indicate their directions (5SP4 and 5SP3 were antisense, 6S and 3SP2 were sense primers). The box marked M shows the possible start point of translation, and the box marked STOP shows the possible end of translation of *AFL1* and *AFL2*. The * indicates an identical nucleotide between *AFL1* and *AFL2* cDNA.

fied a 468 bp fragment. The PCR was performed with AmpliTaq Gold (PE Biosystems, Japan) and was first incubated at 95 °C for 10 min, then incubated by a stepped program (94 °C, 60 s; 62 °C, 60 s; 72 °C, 120 s) for 40 cycles. A 5 μl portion of the reaction mixture was separated on a 1.5% agarose gel and stained by ethidium bromide.

Plant transformation

The *AFL1* and *AFL2* cDNA were amplified between *AFLF* and *AFL1R* or *AFL2R*, respectively (see Figure 1). Then each amplified cDNA was blunted and *Xba*I linkers were ligated both ends, cloned into a pIG121Hm binary vector at the *Xba*I site. The vec-

tor contained the 35S promoter and terminator, as well as the neomycine phosphotransferase and hygromycin gene as selective markers (Ohta *et al.*, 1990). Then sense or anti-sense directional insertion clones were selected. Sense directional clones were named *AFL1S* and *AFL2S*; on the contrary, *AFL2* inserted at antisense direction was named *AFL2AS*. From our EST project on apple flower development, the *C045* clone, which is 1.6 kb in length with a homeobox domain, has high homology with the carrot gene (Kawahara *et al.*, 1995). The *C045* was inserted into pIG121Hm vector, as with *AFL* genes. An *Agrobacterium tumifaciens* GV3101 strain (Van Larebeke *et al.*, 1974) was used for the transformation of the *A. thaliana* Columbia ecotype. *A. thaliana* plants greenhouse-grown for two weeks were transformed by the *in planta* method (Clough and Bent, 1998). Resultant seeds were planted on a 1/2MS culture medium containing kanamycin (20 μg/ml) and hygromycin (20 μg/ml) as selective antibiotics. The antibiotic-resistant plants were maintained as transgenic lines, and their flower phenotypes were observed under long-day conditions (16 h light/8 h dark).

Northern analysis of transgenic plants

Total RNA was isolated from rosette leaves of transgenic *Arabidopsis* grown for 3–4 weeks under the long-day conditions. The isolation method was the same as described above. An equal amount (1 μg) of total RNA from each plant line was electrophoresed with 0.8% agarose gel and blotted onto a nylon membrane (Hybond N+, Amersham). Hybridization was performed with a DIG-labeled RNA probe using the method of Kotoda *et al.* (2000). The resultant images were detected and analyzed with the LAS-1000 image analyzer.

Results

Cloning and sequence analyses of AFL1 *and* AFL2

A comparison of *Arabidopsis LFY* (Weigel *et al.*, 1992), snapdragon *FLO* (Coen *et al.*, 1990) and other homologues (poplar, *PTLF*; pea, *PEAFLO*; petunia, *ALF*; and tomato, *TOFL*) (Hofer *et al.*, 1997; Souer *et al.*, 1998; Molinero-Rosales *et al.*, 1999; Rottmann *et al.*, 2000) showed the presence of several conserved regions. Two of these regions, QREHPFIV (6S) and WYVPTKLR (10A), were used to design degenerate oligonucleotide primers for RT-PCR (Kotoda *et al.*, 2000). Using these primers with cDNA prepared from a floral shoot apex of apple, we obtained a PCR-amplified fragment (440 bp) containing both primers and other conserved sequences. This 440 bp fragment was named *AFL400 (apple FLORICAULA LEAFY* homologue). To obtain a full-length *AFL* cDNA, several primers for 5′/3′ RACE were designed (Figure 1). Two kinds of *AFL* cDNA were obtained by this RACE method. The 5′ common primer (*AFLF*) was designed in the 5′ non-coding region, and the 3′ primer (*AFL1R* or *AFL2R*) was designed in the 3′ non-coding region; each region had a specific sequence. The two kinds of amplified 1.4 kb fragments were sequenced and named *AFL1* and *AFL2*. The coding regions of *AFL1* and *AFL2* showed 90% homology to each other, but the 3′ non-coding regions had less than 60% homology. *AFL1* had a 9 bp 5′ non-coding region and a 176 bp 3′ non-coding region upstream of the poly(A) tail, then a 1230 bp coding region encoding 410 amino acids potentially. *AFL2* had a 23 bp 5′ non-coding region and a 181 bp 3′ non-coding region, and *AFL2* cDNA also had a 1230 bp region encoding 410 amino acids. Their deduced amino acid sequences indicated the same length, but two gaps existed (Figure 2). The deduced amino acid sequence of *AFL1* or *AFL2* cDNA has, respectively, 71.5% or 72.9% homology with *PTLF*, 71.1% or 72.3% with *PEAFLO*, 71.9% or 72.9% with *ALF*, 69.7% or 70.1% with *TOFL*, 68.8% or 69.3% with *FLORICAULA*, 62.0% or 62.8% with *LEAFY*, 63.1% or 64.4% with *ELF1* (Southerton *et al.*, 1998), 53.2% or 55.6% with *RFL* (Kyouzuka *et al.*, 1998), and 46.9% or 46.5% with *NEEDLY* (Mouradov *et al.*, 1998). *LFY, RFL, ALF* and *TOFL* each had a proline-rich region (roughly the first 40 amino acids), but only *AFL2* had an alanine-rich region instead, and both *AFL1* and *AFL2* lacked a proline-rich region.

Genomic analyses and expression patterns of AFL1 *and* AFL2

Genomic DNA extracted from apple was digested with rarely cutting enzymes (*Hin*dIII, *Bam*HI, *Eco*RI), and probed at high stringency with a DIG-labeled *AFL400* amplified PCR method. Both *AFL1* and *AFL2* cDNA sequences corresponding to *AFL400* had no sites of these restriction enzymes. However, only the genomic sequence (including a roughly 900 bp intron, we estimated the length by genome PCR) corresponding to *AFL2* had a site of *Eco*RI. Figure 3 shows that there were four bands with *Hin*dIII digestion, three bands with *Bam*HI and six with *Eco*RI. So, these re-

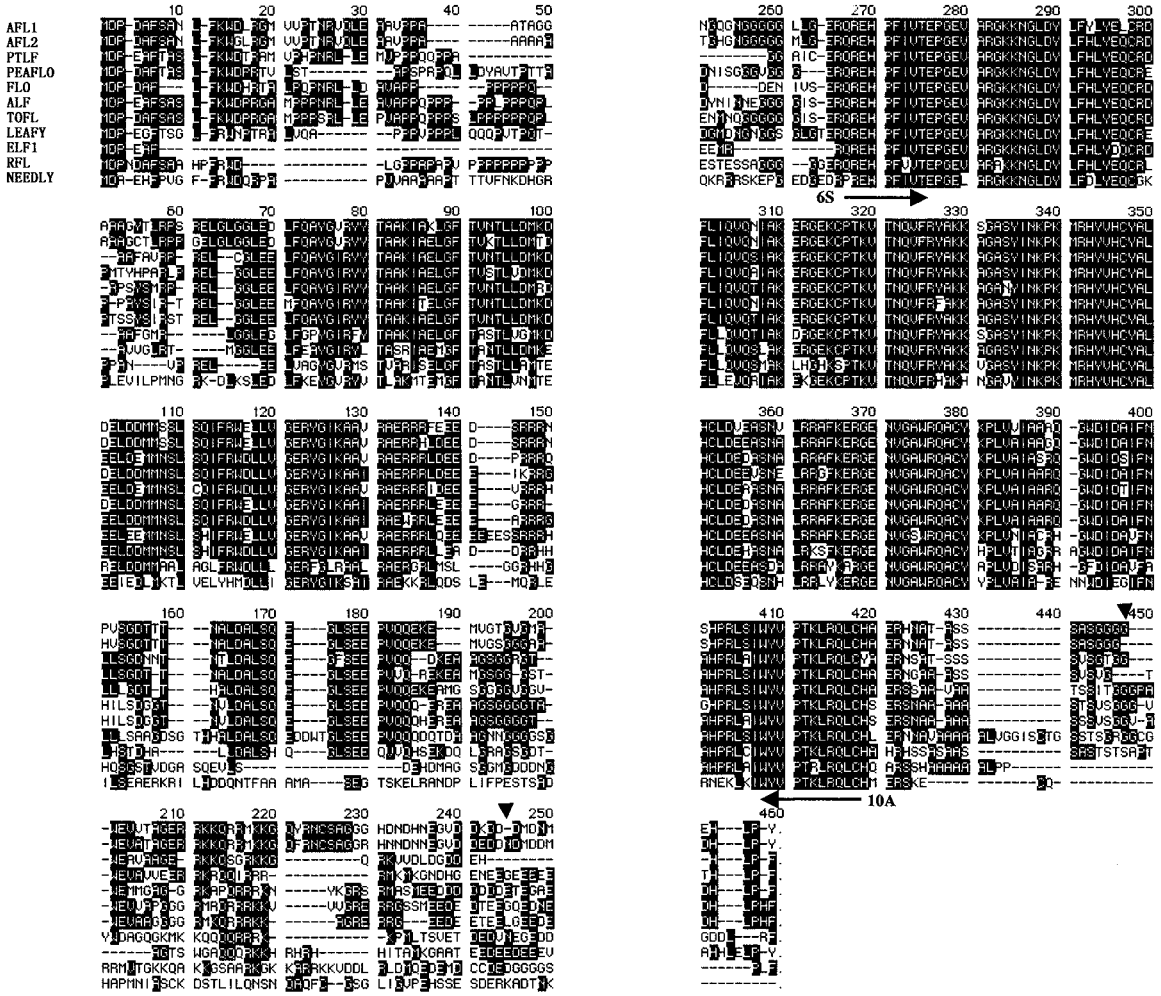

Figure 2. Alignment of deduced apple amino acid sequences corresponding to the *AFL1* and *AFL2* cDNA with *LFY (A. thaliana)*, *FLO* (snap dragon) and homologues from poplar (*PTLF*), pea (*PEAFLO*), petunia (*ALF*), tomato (*TOFL*), *Eucalyptus* (*ELF1*), rice (*RFL*) and pine (*NEEDLY*). Identical residues of the alignments are boxed (white letters on black background). Arrows indicate the positions of primers used to obtain the *AFL400* fragment. Open arrowheads show the gaps. One is at 217N of *AFL2*, the other is at 402G of *AFL1*.

sults indicated that there was at least one homologous gene in addition to *AFL1* and *AFL2*. We cannot detect bands of northern hybridization with an *AFL1*- or *AFL2*-specific probe. Therefore, PCR primers specific to each of *AFL1* and *AFL2* were used to detect the expression patterns in several tissues. One μg of total RNA was inoculated from each flower organ (sepal, petal, stamen and carpel), floral shoot apex (F.S.A.), vegetative shoot apex (V.S.A.), root, stem and leaf. The RT-PCR of *AFL1*-specific primers resulted in amplification of a single band at only F.S.A.; on the other hand, the RT-PCR of *AFL2*-specific primers resulted in a single band in most floral organs, as well as in F.S.A., V.S.A. and root (Figure 4). Increasing the PCR cycle did not change these expression patterns. Both amplified DNA fragments were the expected lengths. Figure 5 shows the expression time course of *AFL1* and *AFL2* on developing floral apexes by RT-PCR as in Figure 4. The numbers on the top picture indicate sampling date (month/day). Jonathan is one of the apple cultivars we used in this experiment. This cultivar's floral apexes vegetatively developed until around the end of June, and typical changes to domed apical meristems were observed microscopically from around the end of August. The transition period, i.e., the period in which no phenotypic changes were observed compared with vegetative growth, appeared to be about a month. *AFL1* began to express clearly at

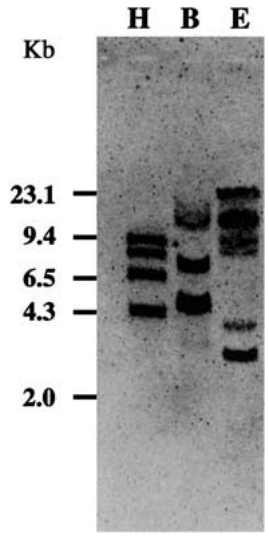

Figure 3. DNA blot analysis of apple genome. Genomic DNA (10 μg) from apples were digested with *Eco*RI (E), *Bam*HI (B) or *Hin*dIII (H), and hybridized with a probe prepared from a PCR-DIG *AFL400* (6S-10A primers). The left numbers indicate molecular size (kb).

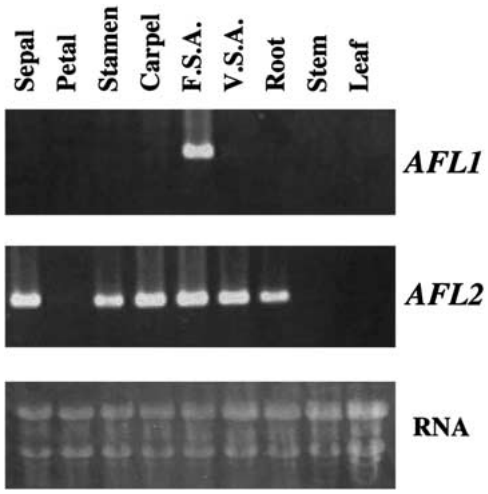

Figure 4. RT-PCR analysis of *AFL1* or *AFL2* RNA in different tissues. The top picture shows PCR amplification with *AFL1*-specific primers. The middle one shows PCR amplification with *AFL2*-specific primers. The bottom one shows agarose gel (1.5%) electrophoresis of RNA samples stained by ethidium bromide from each apple tissue. F.S.V.: floral shoot apex. V.S.A.: vegetative shoot apex.

that interval, but *AFL2* expression was found on all searched days. These results indicated that *AFL1* expression was restricted in a highly regulated manner and the expression occurred just before the phenotypic change of the floral apexes. On the other hand, *AFL2* expression was constitutive and showed no signs coupled with the shift.

Ectopic expression in Arabidopsis

Although the functions of *AFL1* and *AFL2* should be determined by the development of transgenic apple trees, this approach would be actually difficult because to develop transgenic apple trees with flowers would take several years. We therefore used *Arabidopsis* (Columbia) to generate transgenic plants. Both *AFL1* and *AFL2* cDNAs were placed under the control of a CaMV 35S promoter and introduced to *Arabidopsis* by the *in planta* method (Clough *et al.*, 1998). Twenty-five independent transformants by *AFL1* were selected by antibiotics medium, and three lines (1S-1, 1S-2 and 1S-23) showed clear phenotypes of early flowering and solitary flowers arising from rosette axils. In the case of *AFL2* transformation, 19 independent transformants were selected, and four lines (2S-2, 2S-6, 2S-9 and 2S-18) showed more severe phenotypes than did the *AFL1* transformants (Figure 6). The typical phenotypes of *AFL2* transformants were several solitary flowers arising from rosette axils (Figure 6b, c).

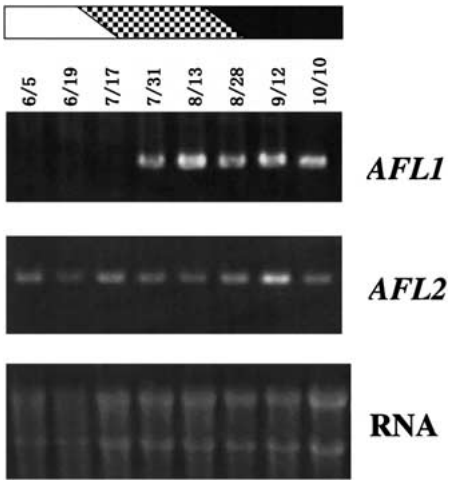

Figure 5. RT-PCR analysis of *AFL1* and *AFL2* RNA at different times. The number on the top picture shows the month/day when each floral shoot apex was collected, from 5 June to 10 October. The picture order is the same as in Figure 4 (top, *AFL1*; middle, *AFL2*; bottom, total RNA). The top bar shows the transition pattern from vegetative to reproductive phase. The left white area shows vegetative state, and the right shaded area shows reproductive state. The middle area with the check pattern shows transition state.

Table 1. Effects on leaf and solitary flower numbers on 35S::*AFL1* or *AFL2* transgenic plants under long-day conditions (16 h L/8 h D).

Plant	Rosette leaves	Range of rosette leaves	Solitary flowers	Range of solitary flowers	*n*
Exp.1					
WT	9.60 ± 1.12	8–12	0	0	15
AFL1S1	8.50 ± 0.75	8–10	0.37 ± 0.74	0–2	8
1S2	8.78 ± 0.89	7–10	0.28 ± 0.72	0–2	14
2S2	5.84 ± 0.80	4–7	4.84 ± 0.68	4–6	13
2S9	7.00 ± 0.92	6–9	5.50 ± 1.06	4–7	8
Exp.2					
WT	9.86 ± 1.12	9–12	0	0	14
AFL1S2	9.36 ± 1.09	7–12	0.81 ± 1.46	0–5	22
2S2	6.00 ± 0.77	5–7	4.45 ± 0.68	3–5	10
2AS2	9.60 ± 0.96	8–11	0	0	10
C045	9.56 ± 0.85	8–11	0	0	30

WT; wild type, *AFL1S*; 35S::*AFL1* transgenic plants lines, *AFL2*S; 35S::*AFL2* transgenic plants lines, *AFL2*AS2; 35S::anti-sense directional *AFL2* transgenic plants line, *C045*; transgenic plants line with homeotic gene from apple under 35S promoter, *n*, plant number. All values are given as the mean and standard deviation. Each experiment was performed independently.

In the case of the 35S::*LFY* transformed *Arabidopsis* (Weigel and Nilsson, 1995), the primary shoot immediately terminated with the formation of a solitary flower. But, the primary shoot of the *AFL2* transformed *Arabidopsis* never terminated and had lateral flowers from axils of cauline leaf instead of secondary shoots (Figure 6c). The solitary flowers often showed abnormal flower phenotypes (Figure 6f–h), and were often observed with five petals and five sepals (Figure 6g). These flowers had fewer stamens instead of more sepals and petals. Other aberrant flowers were observed to have naked embryos and open carpels (Figure 6f), and an *apetala1*-like phenotype that subtended secondary flowers in flower organs (Figure 6g). But most of the aberrant flowers were self-pollinated and normally grown siliques, and the resultant seeds also had the ability to germinate. These transgenic plant lines were used to determine the expression of induced genes. Northern hybridization showed each specific band of *AFL1* and *AFL2* (data not shown). The expression band of *AFL1* was slightly bigger than that of *AFL2*. For each gene, the level of expression had no relationship with the severity of its phenotypes. *AFL1*- or *AFL2*-transformed *Arabidopsis* were self-pollinated, and the phenotypes of offspring were analyzed. The early-flowering and solitary flowers from rosette axils were inherited in the next generation and co-segregated with the kanamycin- and hygromycin-resistant genes. The phenotypes of these transgenic lines grown under long-day conditions are described in Table 1. Both *AFL1* and *AFL2* lines showed early flowering compared to the wild type and control (*AFL2AS* or *C045*) because of their lower numbers of rosette leaves. Further, the *AFL2* line had fewer rosette leaves than the *AFL1* line. Under short-day conditions (8 h light/16 h dark), these tendencies did not change (data not shown). The *AFL2* line had about five solitary flowers per plant, but the *AFL1* line often had no solitary flowers. The numbers of solitary flowers were affected by day length: short-day conditions resulted in far fewer solitary flowers in both the *AFL2* and *AFL1* lines. On the other hand, as the control of *AFL1* and *AFL2* transgenic plants, *AFL24S* (*AFL2* inserted anti-sense direction) into the same vector was introduced to *Arabidopsis*. A total of 18 transgenic plants were obtained and all transgenic lines showed no early flowering and excess solitary flowers. Another control *C045* obtained 16 transgenic lines which showed no differences with wild type.

Discussion

We obtained cDNA clones, *AFL1* and *AFL2*, as apple homologues of *FLORICAULA/LEAFY* by the RACE method. These clones were amplified in a cDNA

library of developing floral buds. RT-PCR analysis showed that *AFL1* was expressed at the floral bud only, and that *AFL2* was expressed at the floral bud and at floral organs, vegetative apex and root (Figure 4). The genomic region hybridized by *AFL400* had one intron-corresponding site, which existed in the *LFY* genome (Weigel *et al*., 1992). Genomic blot analysis showed more than 3 bands with each restriction enzyme (Figure 3). These findings suggested the apple genome has at least three genes homologous to *AFL400*. We reported in a previous paper (Kotoda *et al.*, 2000) that northern hybridization analysis by an *AFL400* probe showed highly hybridized nearly 1.4 kb bands at sepals and leaves. These 1.4 kb bands did not express *AFL1* or *AFL2*, because RT-PCR analysis showed no amplification of leaf tissues by each specific primer (Figure 4). This indicated that at least one other *AFL* family, in addition to *AFL1* and *AFL2*, exists in the apple genome. In addition, there were high levels of homologues expressed in leaves and sepals, yet we had no PCR amplification bands on leaf cDNA by 6S and 10A primers. This suggested that other homologues had less similarity to each other than was found between *AFL1* and *AFL2*. In most plants for which *LFY* homologues were determined, these *LFY*-homologous genes had a single copy in each genome (*Arabidopsis*, snapdragon, pea, tomato, petunia and poplar; Weigel *et al.*, 1992; Hofer *et al*., 1997; Souer *et al.*, 1998; Molinero-Rosales *et al.*, 1999; Rottman *et al.*, 2000). On the other hand, *Eucalyptus* has three homologous genes in the genome, but two of them were found not to be expressed and had stop codons in their coding regions. Hence, only one gene seems to function actually in *Eucalyptus* (Southerton *et al.*, 1998). In gymnosperms, *Pinus radiata* has two homologous genes, *NEEDLY* and *PRFLL*, in the genome (Mouradov *et al.*, 1998; Mellerowicz *et al.*, 1998). *NEEDLY* was expressed during vegetative development, while *PRFLL* was expressed in male cones and not in the females during reproductive development. A search for *LFY* homologues in basal angiosperms and Gnetales showed that the *LFY* family is not always single-copy in diploids (Frohlich and Meyerowitz, 1997). Frohlich further proposed that there is a possibility that angiosperms had a decreased copy number of *LFY* genes from ancestral plants (Frohlich and Parker, 2000). This was consistent with our findings that apple has more than three *LFY* homologues, and *Eucalyptus* had a couple of pseudogenes of possible *LFY* homologues. These plants may have retained the ancestral character of *LFY* genes.

It is believed that apple originated in western Asia and that cultivated apples are complex polyploids (Korban and Chen, 1992). Compared with reported plant species, apple appeared to have more *LFY* homologues in their genomes because of their complex polyploid origin. The origin of this complexity has not been fully understood. Figure 1 shows that *AFL1* and *AFL2* had high homology (90%) in their coding regions, but they were expressed in distinct tissues and times in floral and vegetative development (Figures 4 and 5). To determine the respective functions of *AFL1* and *AFL2*, transgenic *Arabidopsis* by 35S::*AFL1* or 35S::*AFL2* were constructed (Figure 6). Ectopic expression of these genes in *Arabidopsis* caused early flowering and the growth of solitary flowers from rosette axils as with 35S::LFY transgenic *Arabidopsis* under long-day conditions (Table 1) (Weigel and Nillson, 1995; Ratcliffe *et al.*, 1999). 35S::*AFL2* transgenic *Arabidopsis* had solitary flowers from cauline leaf axils instead of secondary shoots (Figure 6c) and more solitary flowers than *AFL1* transgenic lines (Table 1). All 35S::*AFL2* transgenic *Arabidopsis* had solitary flowers from axils of cauline leaves on primary shoot (lf in Figure 6c). But, 35S::*AFL1* transgenic plants had fewer solitary flowers from cauline leaves. Ten transgenic lines by 35S::*AFL1* were observed for 50–60 days under long-day conditions, most lines had secondary shoots from cauline leaves. But, a solitary flower was observed on one axil per couple of transgenic plants (Figure 6i). After longer culture, secondary shoot-like structure arose from the same axil (s in Figure 6i). Such shoot like structure had never been observed with 35S::*AFL2* transgenic plants. This suggested that *AFL1* also had the transition ability of vegetative to reproductive growth like *AFL2* qualitatively, but the effects were much weaker than for *AFL2*. In short-day conditions (8 h light/14 h dark), both transgenic plants showed earlier flowering than the wild type, but there were 20–26 rosette leaves for bolting under the *AFL1* lines, 13–18 rosette leaves under *AFL2*, and 30–35 rosette leaves under the non-transgenic ones (wild type). The numbers of solitary flowers arising from rosette axils decreased with both transgenic lines (data not shown). So the early-flowering and solitary flowers from rosette axils were affected by the photoperiod condition. It was guessed that *AFL1* and *AFL2* worked as orthologues of *LFY* in *Arabidopsis* plants (Weigel and Nilsson, 1995). *AFL2* transgenic lines showed various aberrant flowers (Figure 6f–h), in which solitary flowers had severe phenotypes. Unclosed carpels, higher numbers of petals and

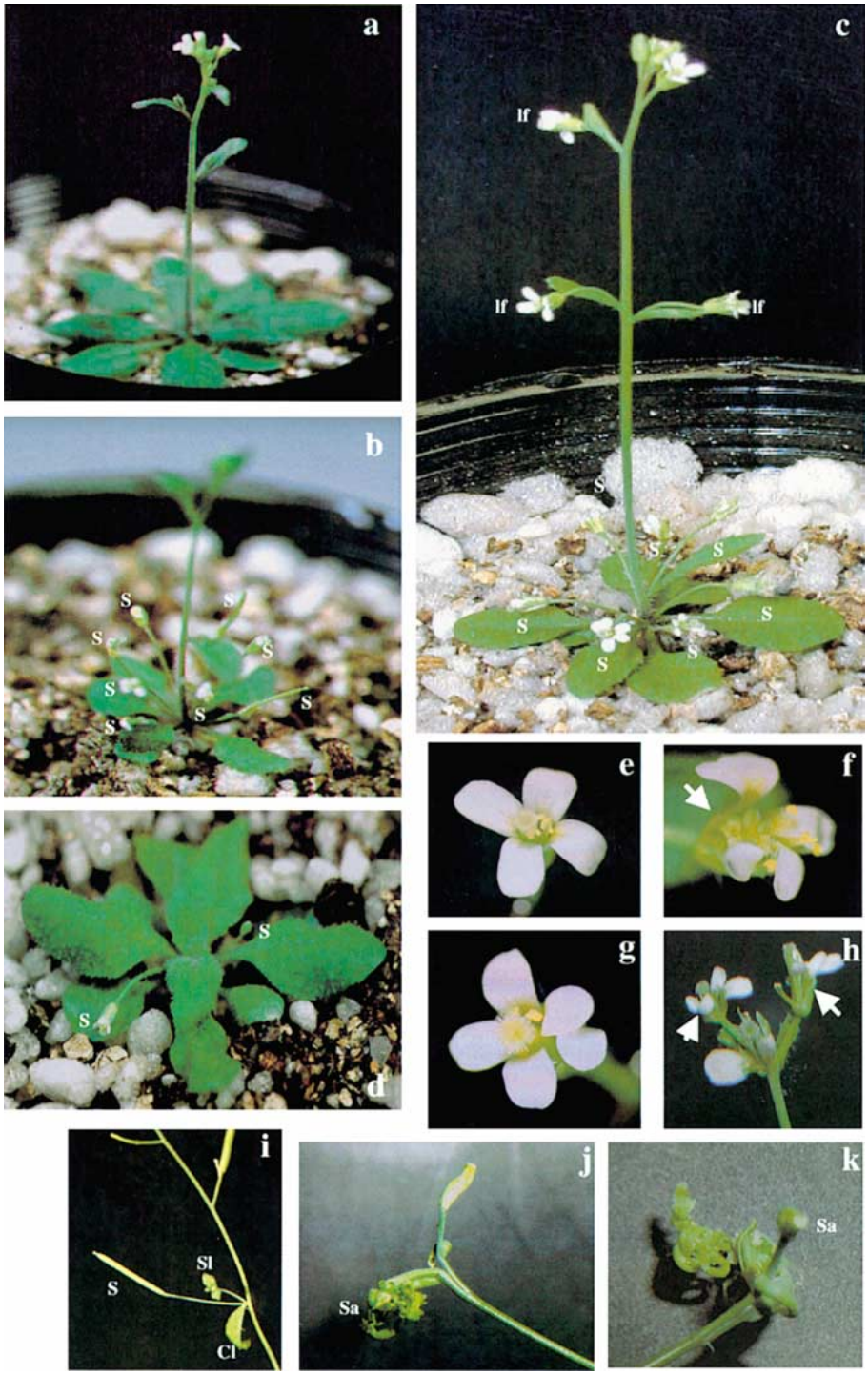

Figure 6. Transgenic *Arabidopsis* plants transformed with 35S::*AFL1* or 35S::*AFL2* grown under a 16 h light/8 h dark photoperiod. a. A phenotype of wild-type *Arabidopsis* (ecotype: Columbia). b. 35S::*AFL2* transformed plant with several solitary flowers (s) arising from rosette axils directly. c. 35S::*AFL2* transformed plant, same as b. The primary shoot grows in an indeterminate manner normally, but the lateral flowers (lf) with axils of cauline leaves were observed. d. 35S::*AFL1* transformed plant. Compared with 35S::*AFL2* plants, the arising solitary flowers were fewer or non-existent, and the flowers tended to arise from lower axils of rosette leaves. e. A normal flower of wild type. f–h. Aberrant solitary flowers observed on 35S::*AFL2* plants. f. The naked embryos (white-head arrow) in unclosed carpels. g. Flower with five sepals and five petals. h. Flower subtends secondary flowers in their axils (white-head arrow) like the *apetala1* mutant of *Arabidopsis* (Alejandra *et al.*, 1992). i. A solitary flower(s) and shoot-like structure (sl) from axil of a cauline leaf (cl) on primary shoot of 35S::*AFL1* transgenic plants. j, k. Fasciated and bending primary shoot apexes (sa) of 35S::*AFL2* transgenic plants.

sepals, and an *apetala1* (Alejandra *et al.*, 1992) like phenotype suggested that ectopic expression of *AFL2* had several effects on internal genes controlling the development of the flower organs. A few reports have made transgenic *Arabidopsis* by *LFY* homologues from other plants (Mouradov *et al.*, 1998; Southerton *et al.*, 1998; Kyouzuka *et al.*, 1998). *NEEDLY* from radiata pine and *ELF1* from *Eucalyptus* transgenic lines showed early flowering and produced solitary flowers from rosette axils and terminal flowers from primary shoots. Those authors mentioned that these phenomena were dependent on photoperiod, as we found the *AFL* transgenic lines to be. However, *AFL* transgenic lines never terminated a primary shoot with a flower. Both *AFL* lines showed indeterminate floral development on their shoots. The 2S-2, *AFL2* transgenic line, which had severe phenotypes (Table 1), was sometimes observed to have primary shoots twisting back on themselves to form a fasciated loop (Figure 6j, k) before recovering and continuing their indeterminate growth. A similar phenomenon was reported on double-transgenic *Arabidopsis* by *LFY* and *Terminal Flower 1* (*TFL1*) (Bradley *et al.*, 1997; Ohshima *et al.*, 1997; Ratcliffe *et al.*, 1999). The two genes have opposite functions in floral development, so their expression under the 35S promoter appeared to result in such aberrant fasciations of the primary shoot because of a lack of harmony between the transition to florescent meristem (*LFY*) and the maintenance of inflorescent meristem (*TFL1*). Thus, it was demonstrated that *AFL2* had a function similar to *LFY* in suppressing *TFL1*. These results have given a clear indication that apple *LFY* homologues *AFL1* and *AFL2* worked as *LFY* orthologues.

AFL1 and *AFL2* had high homology to each other, but their transgenic lines of *Arabidopsis* showed different phenomena (Figures 1 and 6, Table 1). *AFL1* seemed to have weaker effects in *Arabidopsis*. The alignments of *AFL1* and *AFL2* showed there were no partial differences greater than four serial amino acids (Figure 1). This suggested that there is a possibility that such differences in phenomena between their transgenic lines were caused by one amino acid replacement, which might cause dissociation to specific proteins or DNA sequences with the product of *AFL1* or *AFL2*. So, investigation into the effects of such difference on these phenomena could clarify the functional domain of *LFY* orthologues. Lately, MADS-box genes from *Arabidopsis, SEPALLATA1, 2, 3* (*SEP1, 2, 3*), were demonstrated to function as a class of organ identity genes that are required for the development of petals, stamens and carpels. Interestingly, no single mutant ever showed abnormal flowers or abnormal development. But the triple mutant alone showed all flower organs changed to sepaloid ones (Pelaz *et al.*, 2000). Apple *AFL1* and *AFL2* also could work like *SEP* genes in floral development, for which both *AFL* genes may be needed. Unlike the case with *SEP* genes, the temporal expressions of *AFL1* and *AFL2* differed (Figure 5). *AFL2* expressed constantly at all stages, but *AFL1* seemed to express in accordance with floral development. It is possible that the *AFL1* plays a key role in floral development. To test this hypothesis, it will be necessary to determine in what cells the two *AFL* genes are expressed in apple flower bud, or what effects are observed, in transgenic apple flowers by the *AFL* genes under the control of the 35S promoter. The results of such an investigation will clarify the role of each gene in apple floral developments.

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