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Expression Pattern of Homologues of Floral Meristem Identity Genes *LFY* and *AP1* during Flower Development in Apple

Nobuhiro Kotoda,[1] Masato Wada, Sadao Komori,[2] Shin-ichiro Kidou,[3] Kazuyuki Abe, Tetsuo Masuda, and Junichi Soejima

Apple Research Center, National Institute of Fruit Tree Science, Shimokuriyagawa, Morioka 020-0123, Japan

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Abstract. Two apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] homologous fragments of *FLO/LFY* and *SQUA/AP1* (*AFL* and *MdAP1*, respectively) were analyzed to determine the relationship between floral bud formation and floral gene expression in 'Jonathan' apple. The *AFL* gene was expressed in reproductive and vegetative organs. By contrast, the *MdAP1* gene, identified as *MdMADS5*, which is classified into the *AP1* group, was expressed specifically in sepals concurrent with sepal formation. Based on these results, *AFL* may be involved in floral induction to a greater degree than *MdAP1* since *AFL* transcription increased ≈2 months earlier than *MdAP1*. Characterization of *AFL* and *MdAP1* should advance the understanding of the processes of floral initiation and flower development in woody plants, especially in fruit trees like apple.

In contrast to herbaceous plants, apple trees (*Malus sylvestris* var. *domestica*) flower and set fruit only after an extended juvenile phase that may last several years. Thus, it is important to understand the mechanism of floral bud formation because apple is one of the most commercially important tree fruits in the world. However, the genetic factors controlling floral initiation in apples have not been investigated in detail.

In the last decade, several genes related to flower initiation and development have been isolated and the function of these genes is gradually becoming clearer (Liljegren and Yanofsky, 1996; Ma, 1994; Mandel and Yanofsky, 1995; Parcy et al., 1998; Pineiro and Coupland, 1998; Ratcliffe et al., 1998). In snapdragon (*Antirrhinum majus* L.) and arabidopsis [*Arabidopsis thaliana* (L.) Heynh.], *FLORICAULA* (*FLO*)/*LEAFY* (*LFY*) (Coen et al., 1990; Schultz and Haughn, 1991; Weigel et al., 1992; Weigel and Nilsson, 1995) and *SQUAMOSA* (*SQUA*)/*APETALA1* (*AP1*) (Huijser et al., 1992; Irish and Sussex, 1990; Mandel et al., 1992) play central roles in the transition from the inflorescence meristems into floral meristems. Both *FLO* and *LFY* are expressed in the floral meristem prior to floral organ primordia formation and *LFY* in *Arabidopsis* activates *AP1* (Mandel and Yanofsky, 1995). *SQUA* and *AP1* are members of the MADS-box (*MCM1*, *AGAMOUS*, *DEFICIENCE*, and *SRF*, serum response factor) family of a transcription factor, which has a conserved DNA-binding/dimerization region (Coen and Meyerowitz, 1991; Purugganan et al., 1995; Riechmann and Meyerowitz, 1997; Schwartz-Sommer et al., 1990) and they function as both floral meristem and organ identity genes (Bowmann et al., 1993; Guftafson-Brown et al., 1994; Weigel and Meyerowitz, 1994).

In this paper, we describe isolation and characterization of two apple homologue fragments of *FLO*/*LFY* and *SQUA*/*AP1* genes (*AFL* and *MdAP1*) and the relation between expression patterns of *AFL* and *MdAP1*. Furthermore, the morphological changes of apical meristems during flower development in apple were studied in detail.

Materials and Methods

RNA extraction. RNA was isolated from 'Jonathan' apple [age: 14 to 15 years; rootstock: Maruba kaido (*Malus prunifolia* var. *ringo* Asami), location: Morioka, Japan] in 1997 and 1998 by a cetyltrimethylammonium bromide (CTAB)-based method (Chang et al., 1993) modified by Yamamoto and Mukai as follows (not published). About 0.1 g of plant tissue was frozen in liquid nitrogen and then ground to 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) then incubated at 65 °C for 10 min. The solution was extracted with 24 chloroform : 1 isoamyl alcohol (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) then extracted with TE saturated phenol and phenol–chloroform. The aqueous layer was extracted sequentially with chloroform–isoamyl alcohol [24:1 (v/v)] then RNA in the aqueous phase precipitated with 0.1 volume 3 M sodium acetate and 2.5 volumes 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).

Light microscopy. Shoot apices at each developmental stage were fixed in FAA (1.8% formaldehyde, 5% acetic acid, and 45% ethanol) for 24 h at 24 °C. The fixed shoot apices were dehydrated with an ethanol series and embedded in Paraplast plus (Sigma Chemical, St. Louis, Mo.). Longitudinal sections (10 μm) were prepared for morphological observation with a rotary microtome (Yamato Kohki, Asaka, Japan). Sections were stained with 0.5% hematoxylin, 1.0% safranine, and 1.0% fast green FCF. The samples were dehydrated with ethanol transferred to xylene and mounted with Entellan Neu (Merk Chemical, Darmstadt, Germany). The sections were viewed through a Nikon Labophot YF microscope (Nikon, Tokyo, Japan) and photographs were taken using Fujichrome Sensia II 36 film (Fujifilm, Tokyo, Japan).

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[1]To whom reprint requests should be addressed.

[2]Current address: Okinawa Subtropical Station, JIRCAS, Maesato, Ishigaki 907-0002, Japan.

[3]Associate professor, Cryobiosystem Research Center, Faculty of Agriculture, Iwate University, Ueda, Morioka 020-8550, Japan.

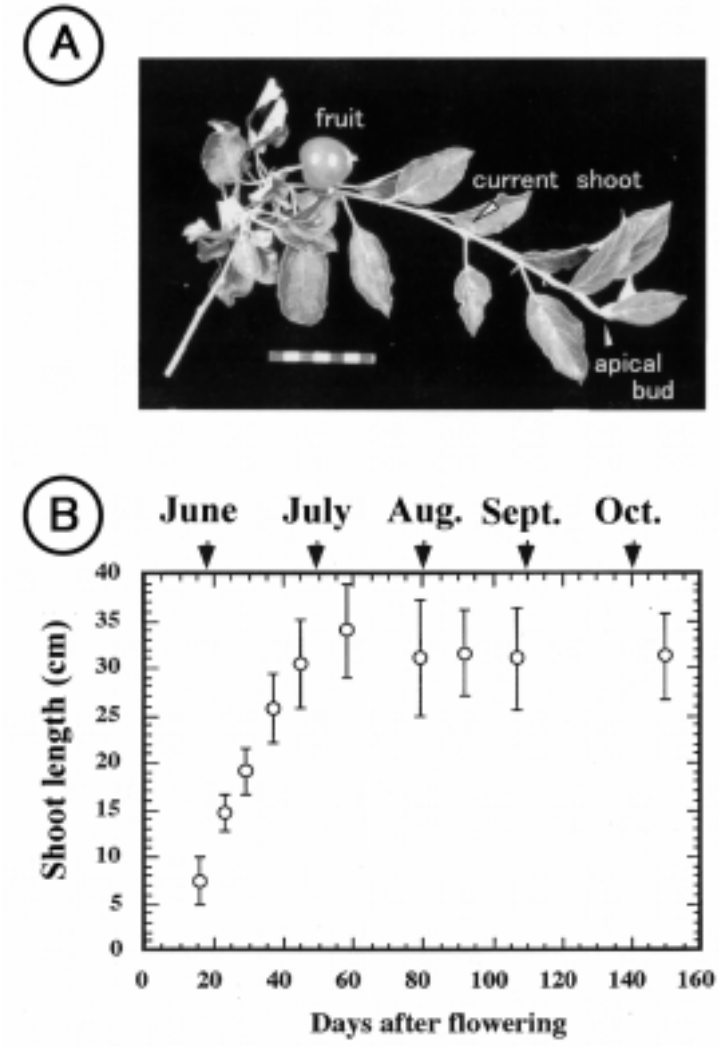

Fig. 1. Growth of current shoot of 'Jonathan' apple from the cluster base. (**A**) Current shoots from the cluster base were collected for measuring shoot lengths and the total RNA was extracted from apical buds. Scale bar = 7 cm. (**B**) The lengths of 40 to 50 shoots were measured after flowering through October. Population of apical buds was synchronized at each developmental stage. Vertical bars = SD.

GENE CLONING. Homologue fragments of *FLO*/*LFY* and *SQUA*/*AP1* were amplified by reverse transcription (RT)-polymerase chain reaction (PCR) from the apices of young floral buds and sepals of mature floral buds in 'Jonathan' apple, respectively.

Two oligonucleotides (5' primer 5'-CAGAGGGAGCAT-CCGTTTATCGTAAC-3' and 3' primer 5'-GACGC/AAGCTTT/GGTT/GGGA/GACATACCA-3') corresponding to the conserved domains in the coding sequence of *FLO* in *Antirrhinum* and *LFY* in *Arabidopsis*, were used to amplify a homologous sequence from apples for the RT-PCR under the following reaction conditions: 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. A 447 base pair (bp) long fragment was amplified and cloned into the pBluescript II SK+ (Stratagene, La Jolla, Calif.) vector. The sequences of one clone of each fragment containing an insert in the pBluescript II SK+ were analyzed by dideoxy methods using a Thermo Sequenase premixed cycle sequence kit according to the manufacturer's instructions (Amersham, Buckinghamshire, U.K.) and universal primers in the Bluescript vectors on a Hitachi SQ5500 automated sequencer (Hitachi, Tokyo, Japan).

Four degenerate oligonucleotides (5' primers 5'-AAA/GGGIAAA/GT/CTITTT/CGAA/GTA-3' and 5'-GAA/GCAA/GCAA/AT/CTIGAT/CACIGC-3', 3' primers 5'-TTT/CTGT/CTCT/CTGA/G/TATIGCT/CTT-3' and 5'-GCIGCA/GAAA/GCAICC-IAA/G-3') were used to amplify homologous sequences of *SQUA*/*AP1* from apple cDNA for the RT-PCR using the following reaction conditions: 40 cycles of 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C. Two overlapping 110- and 567-bp fragments were amplified and cloned into pBluescript II SK+ vectors. The sequences of two clones of each fragment containing an insert in pBluescript II SK+ were analyzed as described above.

RNA BLOTTING ANALYSIS. RNA was isolated from leaves, leaves of tissue cultures of shoots, stems, roots, whole flower buds at various developmental stages, and sepals, petals, stamens, and carpels of 'Jonathan' apple. RNA was separated on a 1.2% agarose gel containing 5.0% (v/v) formaldehyde. Total RNA (10 μg) was loaded per lane. The gels were blotted onto Hybond-N nylon membrane (Amersham). For *AFL* , hybridization was done with DIG-labelled RNA probes, which were prepared by in vitro transcription using the DIG RNA labelling kit (Boehringer Mannheim, Mannheim, Germany) according to the protocol provided by the manufacturer. The pBluescript II SK+ containing a 447-bp fragment of *AFL* was linearized and used as a template to make probes. Hybridization and washing were performed according to a standard protocol (Boehringer Mannheim). For *MdAP1*, the 567-bp fragment of the coding region of *MdAP1* was used as a template to make probes. Hybridization was done with ^{32}P-labelled cDNA probes at 55 °C in 1 M NaCl, 10% dextran sulfate, and 1% SDS for 12 h followed by one rinse in 2× SSC and three washes in 2× SSC and 0.1% SDS and one wash in 2× SSC. RNA blot analysis probed with *MdMADS*, which contains a 75-bp fragment in MADS-box genes in apple (Sung and An, 1997), was performed as a control to *MdAP1* expression. The pBluescript II SK+ vector containing a 75-bp fragment in MADS-box genes in apple 'Jonathan' amplified by RT-PCR with two oligonucleotides (5' primer 5'-CGTCAAGTCACT-TTTTGCAAACGT-3' and 3' primer 5'-AGCATCACAGAG-AACAGAGAGC-3') were used to generate antisense DIG-labeled RNA probes.

Results and Discussion

MORPHOLOGICAL OBSERVATION OF FLOWER DEVELOPMENT IN APPLE. In apple, initiation of flower primordia for the following year occurs 3 to 6 weeks after bloom or after cessation of shoot growth (Buban and Faust, 1982). We collected current shoots from cluster bases after bloom and measured the length every 2 weeks from June to October to determine the time of transition from the vegetative to the reproductive phase in 'Jonathan' apple (Fig. 1A). To determine the morphological changes of apical meristems during flower development, sections of apical buds were prepared (Fig. 2).

In 'Jonathan' apple, growth of most current shoots from the cluster base ceased in late June in Morioka, Japan (Fig. 1B). From this observation, it appears the transition from the vegetative phase to the reproductive phase occurred in late June.

Microscopic examination showed that the apical meristem was a narrow, pointed dome on 3 July (Fig. 2A), slightly domed on 17 July (Fig. 2B), and was clearly broadened by 31 July (Fig. 2C). It appears that initiation of floral bud formation occurs in the middle of July in Morioka. Similar results have been reported by Osanai et al. (1990) and Suzuki et al. (1989).

In the middle of September, the apical meristem domed considerably (Fig. 2D), and sepal primordia were formed in early October (Figs. 2E and H). The initiation of stamen primordia occurred subsequently (Fig. 2H). In the middle of December, several flower

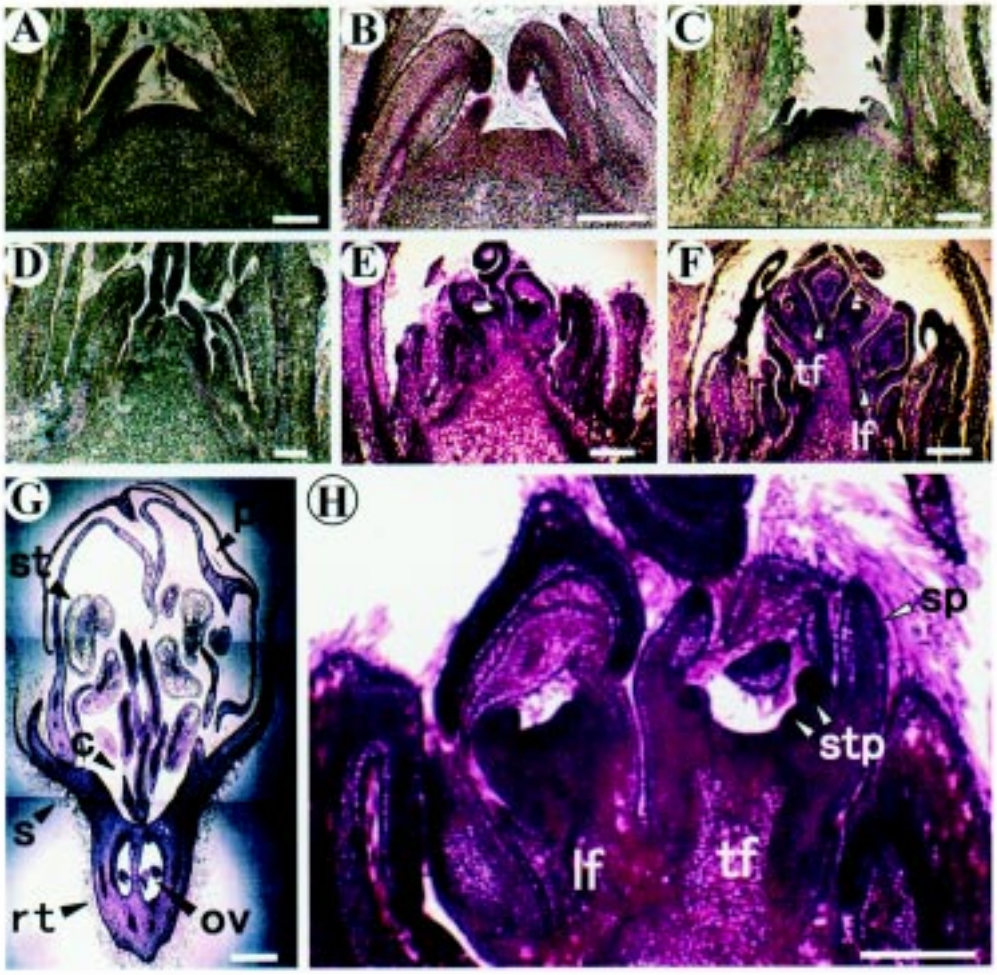

Fig. 2. Morphological changes of apical buds of 'Jonathan' apple. Apical buds from current shoots were fixed in FAA and embedded in Paraplast plus. Longitudinal sections (10 µm) of apical buds were prepared and stained with 0.5% hematoxylin, 1% safranine, and 1% fast green (hematoxylin stains cell walls, cytoplasmic matrices, and plastids purple blue to brownish red, safranine stains lignified cell walls, choromosomes, and nucleoli red, and fast green stains cell walls and cytoplasm green). (**A**) 3 July; (**B**) 17 July; (**C**) 31 July; (**D**) 12 Sept.; (**E**) 10 Oct.; (**F**) 22 Dec. (tf = terminal flower and lf = lateral flower); (**G**) 26 Apr. (p = petal, st = stamen, c = carpel, s = sepal, rt = receptacle, and ov = ovule); (**H**) flower primordia on 10 Oct. (sp = sepal primordia, stp = stamen primordia, lf = lateral flower, and tf = terminal flower). Scale bars = 200 µm (A, B, C, D, H), 400 µm (E, F), and 1000 µm (G).

primordia were visible (Fig. 2F). The floral organs expanded rapidly after the removal of dormancy and were fully differentiated in late April before bloom (Fig. 2G). In general, the inflorescence of apple consists of a terminal flower and four lateral flowers (Fig. 2H). The terminal flower usually grows and opens earlier than lateral flowers.

PCR CLONING AND SEQUENCE ANALYSIS OF *FLO/LFY* AND *SQUA/AP1* HOMOLOGOUS FRAGMENTS IN APPLE. *LFY* and *AP1* are required for floral differentiation in *Arabidopsis* (Mandel et al., 1992; Weigel et al., 1992). Homologous fragments of *FLO*/*LFY* and *SQUA*/*AP1* were amplified by RT-PCR from the apices of young floral buds and sepals of mature floral buds in apples, respectively, to analyze regulation of these two genes during flower development of 'Jonathan' apple.

Two oligonucleotides corresponding to the conserved domains in the coding sequence of *FLO* in *Antirrhinum* and *LFY* in *Arabidopsis* were used to amplify a homologous sequence from apples (Fig. 3A). A 447-bp fragment was amplified and cloned into the pBluescript II SK+ (Stratagene) vector. In addition, four degenerate oligonucleotides were used to amplify homologous sequences of *SQUA*/*AP1* from apple cDNA by RT-PCR (Fig. 3B). Two overlapping 110- and 567-bp fragments were amplified and cloned into the pBluescript II SK+ vectors.

The identity of *AFL* (Apple Floricaula/Leafy) at the nucleotide level was 82% with *FLO* (Coen et al., 1990) and 78% with *LFY* (Weigel et al., 1992). At the amino acid level, the identity was 91% and 89% with *FLO* and *LFY*, respectively (Fig. 3A). The identity of *MdAP1* (*Malus sylvestris* var. *domestica AP1*) at the nuculeotide level was 68% with *SQUA* (Huijser et al., 1992) and *AP1* (Mandel et al., 1992). At the amino acid level, the identity was 63% and 60% with *SQUA* and *AP1*, respectively (Fig. 3B). The deduced protein sequence of *MdAP1* showed higher homology to *SQUA*/*AP1* than any other MADS-box proteins in I (a region between MADS-box and K-box) and K-box regions (Riechmann and Meyerowitz, 1997). The homology with the corresponding *Antirrhinum* and *Arabidopsis* sequences (Fig. 3) suggest that the fragments are derived from the *FLO*/*LFY* and *SQUA*/*AP1* homologues in apples. Therefore, they were named *AFL* and *MdAP1*. In addition, *MdAP1* was identical to the corresponding sequence of MADS-box gene, *MdMADS5*, which was isolated recently from 'Granny Smith' apple (Yao et al., 1999). Difference between the *MdAP1* and *MdMADS5* gene was the substitution of only a few nucleotides in the 3' noncoding region.

EXPRESSION PATTERN OF *AFL* AND *MdAP1* DURING FLOWER DEVELOPMENT. The 447- and 567-bp fragments were used to analyze the expression of both *AFL* and *MdAP1* in the apices of apple at different developmental stages by generating antisense digoxigenin (DIG)-labeled RNA and ^{32}P-labeled cDNA probes, respectively. RNA blot analyses were performed on the total RNA isolated from shoot apices of 'Jonathan' apple during flower development. The *AFL* mRNA was detected in all stages of flower development and weakly detected in vegetative shoots in June (Fig. 4A, 6.19V). It increased from mid-August to mid-November, with a slight decrease in December. The *MdAP1* mRNA in the floral meristem, on the other hand, was first detected in mid-October, when expression of *LFY* was high, and increased drastically until the following April with the formation of floral organs (Fig. 4B). By contrast, *MdMADS* mRNA was detected at a very early stage as well as a late stage (Fig. 4C). These results agree with the model that *LFY* is expressed earlier than *AP1* and regulates *AP1* in *Arabidopsis*. However, *AFL* is expressed weakly in the apical buds of water sprouts which are constantly in a vegetative phase and never flower (Fig. 4A, 6.19V).

EXPRESSION OF *AFL* AND *MdAP1* IN DIFFERENT TISSUES OF APPLE. Expression patterns of *AFL* and *MdAP1* were analyzed in various

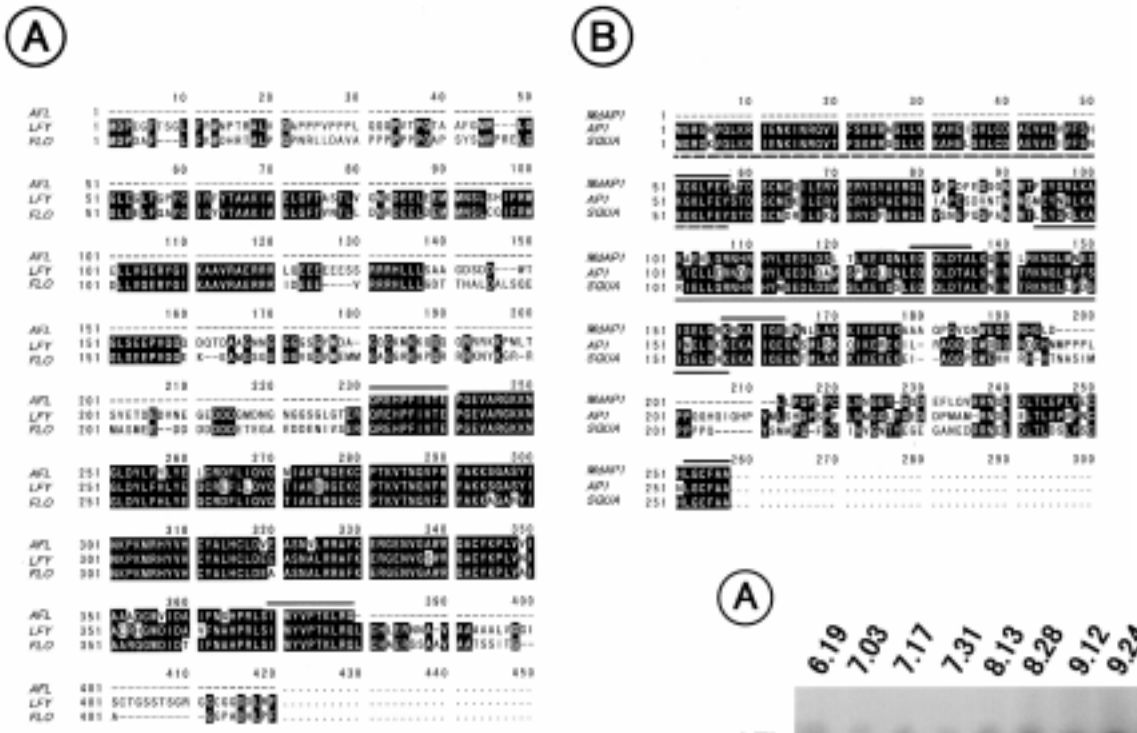

Fig. 3. Comparison of the *AFL* and *MdAP1* sequences with their homologues in *Arabidopsis* (Mandel et al., 1992; Weigel et al., 1992) and *Antirrhinum* (Coen et al., 1990; Huijser et al., 1992). Amino acids at positions of identity between two or three of these proteins are blocked in black. Black lines above *AFL* and *MdAP1* indicate primer sites for RT-PCR. (**A**) Alignment of the partial *AFL* protein sequence with the *Arabidopsis LFY* and the *Antirrhinum FLO* protein sequences. The partial *AFL* obtained corresponded to 447 bp of the *FLO*/*LFY* coding sequence. (**B**) Alignment of the partial *MdAP1* protein sequence with the *Arabidopsis AP1* and the *Antirrhinum SQUA* protein sequences. The partial *MdAP1* obtained corresponded to the entire coding sequence downstream of the *SQUA*/*AP1* MADS-box. A broken line below *SQUA* indicates MADS-box and a black line indicates K-box.

living tissues by RNA blot hybridization. *AFL* was expressed strongly in the sepals, leaves, and leaves of tissue cultures of shoots but very weakly in the petals, carpels, cotyledons, and stems. There was no expression in roots (Fig. 5A). In *Arabidopsis*, *LFY* is expressed strongly in young flower primordia and cauline leaves, but very weakly in flower primordia at the late stage (Weigel et al., 1992). In *Antirrhinum*, *FLO* shows a similar expression pattern to *LFY* (Coen et al., 1990). In contrast to *LFY* and *FLO*, *AFL* RNA was expressed in mature leaves and mature floral organs including sepals, petals, and carpels.

Expression of *MdAP1* was detected specifically in the sepals (Fig. 5B). In comparison, *AP1* is expressed in the sepals, petals, and pedicels, and *SQUA* in the sepals, petals and bracts. Both eucalyptus (*Eucalyptus globulus* Labill.) *EAP1* and *EAP2* were expressed in the operculum which is a unique organ with sepals and petals fused, and in the receptacle (Kyozuka et al., 1997).

We isolated apple homologue fragments of *FLO*/*LFY* and *SQUA*/*AP1* (*AFL* and *MdAP1*, respectively) and analyzed the expression pattern of these genes. Results showed that the *AFL* gene was expressed in reproductive and vegetative organs such as apical buds of water sprouts and mature leaves. In addition, in situ hybridization probed with *AFL* revealed that the *AFL* transcript was present at high levels in apical meristems and leaf primordia at the beginning of floral bud initiation, whereas it was not detected in apical meristems of vegetative shoots (data not presented). For example, *FLO* and

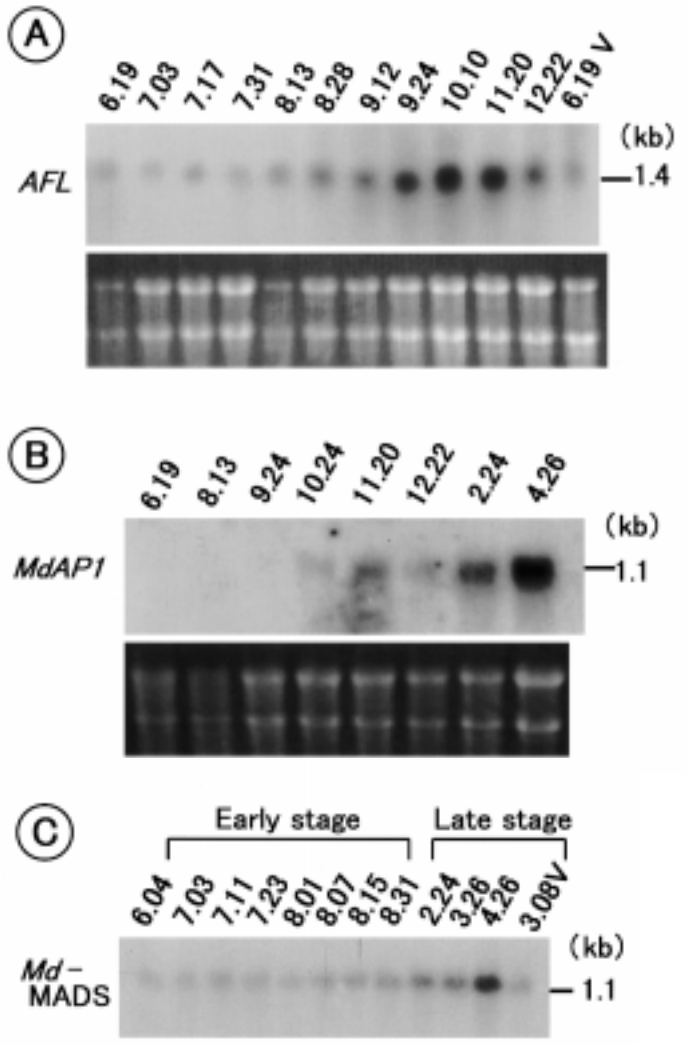

Fig. 4. Northern-hybridization expression pattern of (**A**) *AFL*, (**B**) *MdAP1*, and (**C**) *MdMADS* genes during flower development. The numbers above the lanes indicate dates of harvest (month.day). Equal amounts (10 μg) of total RNA isolated from apical buds were blotted onto Hybond N filters. RNA blot analysis probed with *MdMADS*, which contains a 75-bp fragment in MADS-box genes in apple (Sung and An, 1997), was performed as a control to *MdAP1* expression (Fig. 4C). V = vegetative shoot (water sprout).

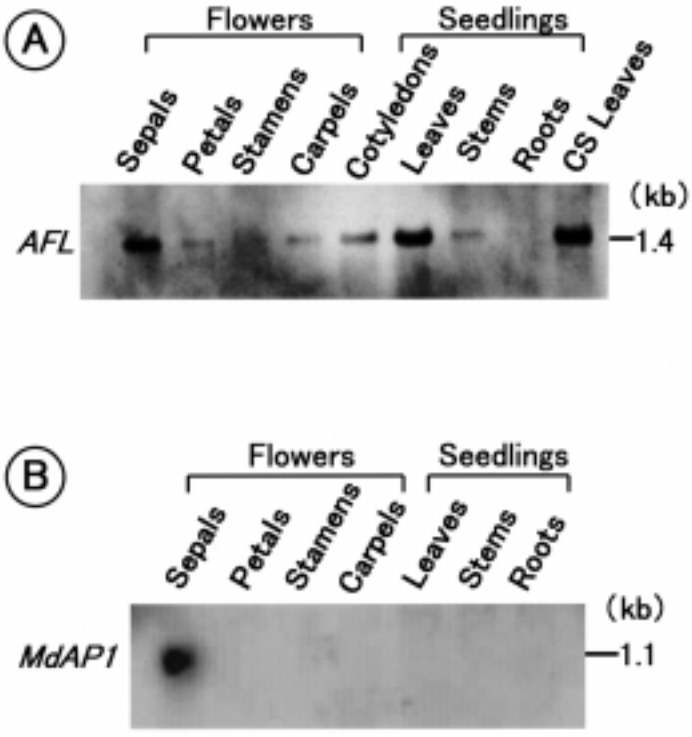

Fig. 5. Northern-hybridization expression pattern of *AFL* and *MdAP1* genes in different organs. (**A**) Equal amounts (10 µg) of total RNA isolated from flowers (sepals, petals, stamens, and carpels), seedlings (cotyledons, leaves, stems, and roots) and leaves of cultured shoots (CS leaves) were subjected to northern analysis. Hybridization was done with DIG-labeled RNA probes, which was transcribed from the recombinant Bluescript plasmids containing a 447-bp fragment of *AFL* with DIG-UTP as described by the manufacturer. (**B**) Equal amounts (10 µg) of total RNA isolated from flowers (sepals, petals, stamens, and carpels) and seedlings (leaves, stems, and roots) were subjected to northern analysis. Hybridization was done with ^{32}P-labeled cDNA probes. A *MdAP1* cDNA of the 567-bp fragment obtained was used as a probe. Values on the right indicate the size of transcripts (kb: kilobase).

LFY are expressed in bract and cauline leaf primordia, respectively. Blazquez et al. (1997) reported that *LFY* is expressed extensively during the vegetative phase. The pea (*Pisum sativum* L.) *FLO*/*LFY* homologue, *PEAFLO* is expressed strongly in leaves, inflorescences, and lateral shoot primordia and in floral organ primordia (Hofer et al., 1997). In impatiens (*Impatiens balsamina* L.), *Imp-flo* homologous to *FLO/LFY* is transcribed in all floral organs including bracts, petals, stamens, and carpels (Pouteau et al., 1997). Compared with the examples described above, the expression pattern of *AFL* in apple resembles that of *FLO*, *LFY*, *Imp-flo*, and *PEAFLO*. *AFL* mRNA, however, was also detected in fully differentiated tissues such as mature leaves and mature floral organs including sepals, petals, and carpels. This result suggests that *AFL* functions during leaf and flower morphogenesis at later developmental stages as well as at the early developmental stage of floral bud formation.

RNA blot analysis probed with *MdMADS*, which contains a 75-bp fragment in MADS-box genes in apple (Sung and An, 1997), was performed as a control to *MdAP1* expression (Fig. 4C). This probe is considered to detect all MADS-box genes in apple. The result showed that *MdMADS* mRNA was detected at early and late stages in apical buds. On the other hand, *MdAP1* was expressed specifically in sepals and showed a similar expression pattern to *SQUA*, *AP1*, *EAP1*, and *EAP2* in that their expression was also detected in sepals or sepal-like organs. While this manuscript was in preparation, Yao et al. (1999) reported that *MdMADS5*, identical to *MdAP1* in corresponding sequences, was expressed strongly in the receptacle, cortex, and skin of apple fruit. *MdAP1* expression was first detected in mid-October when the sepal primordia began to develop and *LFY* expression was high in apical buds. It has been proposed that *FLO*/*LFY* interacts with and activates *SQUA*/*AP1*, so *AFL* may also activate *MdAP1*. Because there was a delay of ≈3 months in *MdAP1* gene expression after floral initiation, *MdAP1* may not trigger floral initiation directly, but it is certain that the gene is involved in sepal and fruit formation. Based on the experimental results, *AFL* may be involved in floral induction to a greater extent than *MdAP1* since *AFL* showed increased transcription ≈2 months earlier than *MdAP1*.

Characterization of *AFL* and *MdAP1* should advance the understanding of the processes of floral initiation and flower development in woody plants, especially in tree fruits like apple. A recent study has shown that an aspen hybrid of two poplar species (*Populus tremula* L. x *P. tremuloides* Michx.), may be induced to generate flowers after only 5 months growth following insertion of the *LFY* gene into cells regenerated from aspen stem segments (Weigel and Nilsson, 1995). Kyozuka et al. (1997) showed that *Eucalyptus EAP1* and *EAP2* have homology to *AP1* and transgenic *Arabidopsis* plants expressing either *EAP1* or *EAP2* are early-flowering and have an increased number of flowering stems. One outcome of plant biotechnology could be reduction of generation time by introducing floral genes like *LFY*, *AP1*, and their homologues through transgenic approaches, which could be useful for hastening breeding strategies.

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