Molecular studies on the flower development in apple (*Malus* × *domestica* Borkh.)

リンゴの花芽形成に関する分子生物学的研究

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Abbreviations

AP1:	*APETALA1*
AFL:	apple homologue of *FLORICAULA/LEAFY*
BA:	6-benzyl-aminopurine (MW=203.2)
bp:	base pairs
CAL:	*CAULIFLOWER*
CaMV;	cauliflower mosaic virus
cDNA:	DNA complementary to RNA
CO:	*CONSTANS*
Col:	Columbia
CTAB:	cetyl trimethyl ammonium bromide
cv:	cultivar
DIG:	digoxygenin
dNTP:	deoxyribonucleotide
EDTA:	ethylenediamine tetra-acetic acid
EtBr:	ethidium bromide
g_n:	gravitation
FLO:	*FLORICAULA*
FT	*FLOWERING LOCUS T*
GUS:	β -D-glucuronidase
IBA:	indole-3-butyric acid (MW=225.2)
kb:	kilobase
LA-PCR:	long and accurate PCR

LB agar/broth	Luria-Bertani agar/broth
LD:	long days
LFY:	*LEAFY*
M:	molarity
MOPS:	3-morpholinopropanesulfonic acid
mRNA:	messenger RNA
MS medium:	Murashige and Skoog's medium
MW:	molecular weight
Na-Pi	Sodium-phosphate
*npt*II:	neophosphotransferase
OD:	optical density
PCR:	polymerase chain reaction
pH:	hydrogen ion exponent
poly (A+) RNA:	polyadenylated RNA
RACE:	rapid amplification of cDNA ends
RNA:	ribonucleic acid
RNase:	ribonuclease
rpm:	revolution per minute
RT-PCR:	reverse transcription-polymerase chain reaction
SD:	short days
SD:	standard deviation
SDS:	sodium dodecyl sulfate
SSC:	saline sodium citrate
SSPE:	saline sodium citrate
SQUA:	*SQUAMOSA*

T-DNA:	tranfer DNA
TAE:	Tris-acetate-EDTA electrophoresis buffer
TE buffer:	10 mM Tris-HCl (pH 7.5) with 1 mM EDTA
TFL1:	*TERMINAL FLOWER1*
Tris:	tris (hydoxymethyl) ammonium
TDZ:	thidiazuron (MW=220.2)
UV:	ultraviolet
WT:	wild-type
w/v:	weight/volume
w/w:	weight/weight

Abstract

Fruit trees such as apple (*Malus*×*domestica* Borkh.) flower and set fruit only after an extended juvenile phase lasting several years. Therefore, the long juvenile phase has been limiting the efficient breeding of fruit trees. In the fruit industry, it is important to accelerate flowering by reducing the juvenile or vegetative phase of the trees after planting in order to facilitate the earliest possible production of fruit. Various practical techniques have been considered to accelerate the flowering and fruiting of seedlings in apple breeding, but it is difficult to reduce the juvenile phase of apple seedlings to less than 4 years under normal growing conditions. Thus, studying the juvenility, floral induction, and flower development in apple and developing the efficient apple transformation system will advance both breeding and genetic studies. Elucidation of basic mechanisms of flower development in apple will help the development of the fruit industry. However, the genetic and environmental factors that control flower induction and flower development in apple have yet to be investigated. In this study, apple genes involved in flowering or juvenility have been isolated and characterized using transgenic *Arabidopsis* and transgenic apple.

(1) Isolation and expression analysis of apple genes *AFL* (apple *FLORICAULA/LEAFY*) and *MdAP1* (*Malus* x *domestica AP1*)

Two apple homologous fragments of *FLORICAULA* (*FLO*)/*LEAFY* (*LFY*) and *SQUAMOSA* (*SQUA*)/ *APETALA1* (*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 in apical buds increased from late June about 2 months earlier than *MdAP1*.

(2) Characterization of transgenic *Arabidopsis* expressing *MdAP1*

An apple gene *MdAP1* was introduced into *Arabidopsis* using *Agrobacterium tumefaciens* EHA101 to examine how the gene affects flowering and whether it may be effective in producing transgenic apple that flowers early. Five of the 15 primarily transformed (T_0) plants flowered 5 to 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_0 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 it might not be involved directly in floral induction in apple.

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

We cloned *MdTFL1* (*Malus domestica TFL1*), a gene homologous to *TERMINAL FLOWER 1* (*TFL1*) that suppresses the floral meristem identity genes *LFY* and *AP1* and maintains the inflorescence meristem in *Arabidopsis*. *MdTFL1* mRNA was expressed preferentially in apple vegetative tissues such as apical buds, stems, and roots of seedlings, and expression peaked in early July in apical buds, about two weeks prior to floral bud differentiation. Transgenic *Arabidopsis* expressing *MdTFL1* flowered noticeably later than wild-type plants and exhibited a phenotype similar to that of transgenic *Arabidopsis* overexpressing *TFL1*. These results suggest that *MdTFL1* is involved in the maintenance of the vegetative phase in apple and that it functions analogously to *TFL1*.

(4) Analysis of transgenic apple 'Orin' expressing *MdTFL1* antisense gene

To clear the function of *MdTFL1* in apple, we produced transgenic apples expressing *MdTFL1* antisense RNA. One of them flowered only 8 months after grafting to rootstocks,

whereas the non-transformed control plants have not flowered in less than 6 years. As expected, the expression of the endogenous *MdTFL1* was suppressed in the transgenic lines that showed precocious flowering. In addition, the expression level of the transgene is correlated with the reduction of the juvenile phase. These findings confirm that *MdTFL1* functions like *TFL1* and that *MdTFL1* maintain the juvenile and vegetative phases in apple. Flower organs of the transgenic apples were normal in appearance, and a precocious flowering transgenic line set fruits and seeds. Interestingly, some flowers of the transgenic apple developed without undergoing dormancy. The expression of *MdTFL1* in apple affected flower development as well as flower induction. The future use of these techniques should be of advantage in breeding, crop production, and basic research such as molecular studies on woody plants, including fruit trees.

Chapter 1

General introduction

1. 1 Taxonomic classification of apple

Apples are members of the genus *Malus*, which is placed in the subfamily *Maloideae* of the family *Rosaceae* (Fig. 1-1). Other members of the *Maloideae* include pears (*Pyrus* L. spp.), quinces (*Cydonia oblonga* Mill.), loquats [*Eriobotrya japonica* (Thunb.) Mgill], medlars (*Mespilus germania* L.) and species of *Amelanchier Aronia*, etc. The subfamily *Maloideae* has a high haploid base chromosome number of x = 17 and is generally considered to be monophyletic when morphological traits, chromosome number and DNA sequence variation from the chloroplast rbcL gene and S-RNase (self-incompatibility) gene are considered. Most recent researchers assign *Malus* species to five sections of the genus based on morphological traits and flavonoid similarities (Fig. 1-1). 1. Section *Malus*, consisting of series *Malus* including European and Asian species (including *M. sieversii* and *M.* x *domestica*), with fruit having five carpels and mostly persistent calyces on the fruit, and series *Baccatae*, containing several Asian species, with fruit consisting of three to five carpels and deciduous calyces. 2. Section *Sorbomalus*, including series *Sieboldianae*, with species native to Japan, series *Florentinae*, series *Kansuenses*, and series *Yunnanenses*. 3. Section *Eriolobus*, containing only *Malus eriolobus* (Poir.) C.K. Schneid, from the eastern Mediterranean. 4. Section *Chloromeles*, containing North American species. 5. Section *Docyniopsis*, containing the species *Malus tschonoskii* (Maxim.) C.K. Schneid., *Malus doumeri* (Bois) and *Malus formosana* Kawak. & Koidz. of Japan, Taiwan and South-East Asia. The common domesticated apple is putatively an interspecific hybrid complex, usually named *Malus* x *domestica* Borkh. (Korban and Skirvin, 1984) or *M. domestica* Borkh. (Phipps et al., 1984).

Besides *M. domestica*, fruits of several other species are consumed fesh or processed or are used for medicinal purposes and plants are used as rootstocks. Many species and interspecific hybrids are used as ornamental plants. The origin and ancestry of the *M.* x *demestica* complex remain unknown. Currently, *Malus sieversii* (Ledeb.) Roem. is hypothesized as the key species in its origin. *M. sieversii* is widespread in the mountains of central Asia at elevations between 1200 and 1800 m. During the late 19th and 20th centuries, *M.* x *domestica* cultivars found or bred in Europe, Russia, North America, New Zealand, Australia and Japan were introduced throughout the world and form the basis for most current commercial apple production.

1.2 Juvenility, flowering, and fruit development in apple

Apple seedlings must pass through an extended juvenile period before they are capable of flowering. In most woody plants, it requires from 1 to 20 years, depending on species. In *Rosaceae* family, it requires from 4 to 8 years for *Malus* spp., 2 to 8 years for *Prunus* spp., and 6 to 10 years for *Pyrus* spp. (Hackett, 1985). Treatments that stimulate flowering, such as the ringing of the bark, scoring, bark inversion, root pruning have little effect on seedlings. Grafting scions from seedling trees onto bearing trees can hasten flowering, but the time gained is only 1 or 2 years. The basic idea to hasten flowering is to grow the seedlings rapidly from the germination stage to the transition to flowering (Aldwinckle, 1975; Visser, 1964) but, only buds near the top of the tree flower. The observation appears to be due to an effect of 'distance from the roots', suggesting that inhibitory compounds produced in the roots move to up the stem and prevent flowering.

The floral buds of apple trees are initiated in the season prior to their unfolding into blossoms. Most flowers on apple are located terminally on short shoots. The exact time when initiation of floral buds occurs in the apple depends on crop load, shoot growth rate, environmental conditions, and cultivars (Childers et al., 1995). Flowers develop from a bourse

bud growing in the axils of leaves (Fig. 1-2). Before flowers can form, a bud must go through a vegetative phase and produce from 16 to 21 appendages which include bud scales, transition leaves, true leaves, and bracts. Once these have been produced, then floral buds can develop laterally in the axils of the true leaves. Typically, four to six flowers will form in each spur (Fig. 1-2A).

Most flower induction occurs in early summer. Initiation begins when the meristem flattens and continues to form sepals, petals, stamens, and pistils centripetally on the apex. In apple cultivar 'Jonathan', growth of most current shoots from the cluster base (bourse) ceased in late June in Morioka, Japan, suggesting that the transition from the vegetative to the reproductive phase occurred in late June. Microscopic examination shows that the apical meristem was slightly domed on mid July, demonstrating 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 most fruit-growing areas, buds become dormant in late summer to early autumn and winter chilling is necessary to permit renewed growth in the following year (Saure, 1985). This is a common occurrence among temperate tree species and enables the tree to withstand the cold winter.

Most apple cultivars require cross-pollination to set fruits. Many important cultivars such as 'Fuji', 'Tsugaru', and 'Jonagold' will not form seeds nor set fruits when their blossoms are self-pollinated. Such cultivars are known as self-unfruitful. If a set of fruit results from self-pollination, the cultivar is said to be self-fruitful. In apple, however, there are no self-fruitful commercial cultivars which meet consumer demand.

1.3 Apple production and breeding

Apples are one of the world's leading fruit crops widely grown both in the Northern and Southern Hemisphere with production of almost 60 million tons (Fig. 1-3; O'Rourke, 2003). Four

apples, 'Fuji', 'Golden Delicious', 'Delicious' and 'Gala' account for about 50% of world production. 'Fuji' is now the leading world apple cultivar with 12.3 million tons, about 20% of world production. 'Delicious' and 'Golden Delicious' are second and third in the world, with 9.3 and 8.8 million tones, respectively. The apple production in Japan amounts to about 0.9 million ton. 'Fuji', 'Tsugaru', 'Jonagold' and 'Orin' account for 55%, 12%, 10% and 8% of the total production in Japan, respectively. The importance of breeding is becoming apparent and four of the world top 10 apples ('Fuji', 'Gala', 'Jonagold', 'Idared') are the products of breeding programs. Most of all Japanese cultivars including 'Fuji', 'Tsugaru' and 'Sansa' resulted from the breeding programs from the 20th century downward. However, the present leading apples are all quite disease susceptible. Thus, the breeding for disease resistance, as well as for improvement of fruit quality such as sugar content, texture, and skin color, have been developed for many years in the world including Japan. Breeding fruit trees such as apple often takes more than 20 years, including periods of cross pollination, seedling selection, and regional trials to produce varieties that meet consumer demands. For example, the 'Fuji' apple, one of the most popular apple cultivars in the world (O'Rourke et al., 2003), first set fruit 12 years after sowing, and it took 23 years before its release as a cultivar (Fig. 1-4; Sadamori et al., 1963). For this reason, in the fruit industry, it is important to accelerate the flowering by reducing the juvenile or vegetative phase of the trees after planting in the orchard and make it produce fruits as early as possible. In apple, grafting the seedlings onto bearing mature trees or dwarfing rootstocks such as 'Malling 9' ('M.9') and 'Malling 27' ('M.27') will usually bring earlier flowering (Visser, 1964, 1973). Thus, the apple cultivar 'Sansa', which was top grafted for the acceleration of flowering in the breeding program at our institute, first set fruits 6 years after sowing, and then released as a cultivar 18 years after crossing (Yoshida et al., 1988).

1. 4 Genetic transformation of apple

In general, genetic transformation of woody plants is more difficult than that of herbaceous plants. Gene transfer into apple was first reported in 1989 by James, and since then, several reviews including apple transformation have been written (Dandekar, 2002; Hammerschlag, 2000; Yao et al., 2000). In the transformation system, *Agrobacterium tumefaciens* harboring a transformation vector is inoculated with subdivided tissue cultured leaves. The outline of apple transformation is shown in Fig. 1-5. In contrast, genetic transformation of *Prunus* species such as peach and cherry has not been established in spite of much effort by a number of researchers. So far, all fruit trees belonging to *Rosaceae* family can not be transformed genetically. For example, genetic transformation of Japanese pear has not developed although that of European pear was reported in the late 1990's (Mourgues et al., 1996; Bell et al., 1999). Genetic engineering offers a powerful adjunct to conventional breeding because it offers single or several genes improvement of existing commercial cultivars and because genes out of the scope of conventional breeding can be introduced (Hammershlag, 2000). Apple cultivars 'Greensleeves' (James, 1989), 'Gala', 'Golden Delicious', and 'Elster' (Puite and Schaart, 1996) were transformed, followed by 'Delicious' and 'Pink Lady' (Sriskandarajah and Goodwin, 1998). Although apple breeding programs using sexual hybridization have generated new apple cultivars with disease resistance, winter hardiness, lower chilling requirements and improved eating qualities, a large number of commercially grown apple cultivars have been derived from chance seedling or from natural mutation occurred in some of the important apple cultivars. In the early 1990s, transgenes introduced into apple were antibiotics resistance genes such as neophosphotransferase gene II (*npt*II) or reporter genes such as β-glucronidase gene (*gus*) for the development of transformation system (De Bondt et al., 1996; Maheswaran et al., 1992; Puite and Schaart, 1996; Sriskandarajah et al., 1994). Thereafter, several apple cultivars were transformed with disease resistance genes encoding attacin E (attE), cecropin (cec), chitinase, glucanase, and sarcotoxin (Liu et al., 2001; Norelli et al., 1994; Soejima, 2000) or transformed

with herbicide resistance genes such as acetolactate synthase gene (a gene resistant to the herbicide Glean^{TM}) and bialaphos resistance gene (*bar*) (Yao et al, 1995). In the late 1990's, biosynthetic pathway genes encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase, and sorbitol-6-phosphate dehydrogenase (S6PDH) were introduced into apple to modify the metabolites related to storage and sugar content of fruits (Defilippi et al., 2005; Kanamaru et al., 2004). In this study, genetic transformation system using a Japanese apple cultivar 'Orin' was adopted because it is one of the economically important cultivars in Japan and relatively easy to be regenerated from the explants. Mature fruits and cultured tissues of 'Orin' apple are shown in Fig. 1-6.

1. 5 Importance of the studies on the mechanism of flower development in apple

Progress of traditional fruit breeding programs are slow because of the long juvenile phase, high levels of heterozygosity and time necessary to adequately evaluate hybrids. Especially, the length of the juvenile phase is inversely related to the breeding efficiency of woody perennials such as fruit trees such as apple. Also, genetic and molecular studies of fruits trees fall behind those of herbaceous plants such as *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), and tomato (*Lycopersicum esculentum* Mill.) because of their long generation time and the difficulty of efficient transformation procedures as well as their large genome and self-incompatibility. Thus, it is important to study the juvenility, floral induction, and flower development in apple and the efficient apple transformation system from the viewpoint to advance both breeding and genetic studies. Consequently, elucidation of basic mechanisms of flower development in apple will help the development of the fruit industry. However, the genetic and environmental factors that control flower induction and flower development in apple have yet to be investigated.

In contrast, the most detailed and comprehensive picture of the molecular mechanisms underlying flower development has been obtained from *Arabidopsis* (Jack, 2004; Levy and Dean,

1998; Mouradov, 2002; Pineiro and Coupland, 1998). The first unifying principle in the flower development field is the ABC model (Bowman et al, 1991; Weigel and Meyerowitz, 1994). This model is very simple and is applicable to a wide range of angiosperm species (Fig. 1-7). The *Arabidopsis* flower consists of four organs that are arranged in whorls. From outside to inside, the flower consists of sepals in whorl 1, petals in whorl 2, stamens in whorl 3, and carpels in whorl 4. The ABC model hypothesizes that three activities, A, B, and C, specify floral organ identity in a combination manner. Shortly, A alone specifies sepals, A+B specifies petals, B+C specifies stamens, and C alone specifies carpels. Throughout the 1990s, the ABC genes were cloned from a wide range of species including apple. Apple genes, *MdMADS2* and *MdAP1* (*MdMADS5*), homologous to *APETALA1* (*AP1*) which is an A class gene, have been isolated and characterized (Kotoda et al., 2000, 2002; Sun et al., 1999; Yao et al., 2000). Mutations in the *PISTILLATA* (*PI*) gene of *Arabidopsis* cause homeotic conversion of petals to sepals and of stamens to carpels (Goto and Meyerowitz, 1994). Interestignly, there are apple mutants such as 'Noblow' and 'Wickson' with flowers similar to those of the *Arabidopsis* pi mutant. Yao et al. (2001) identified that in the apetalous mutant *MdPI* (an apple homologue of PI) has been mutated by a retrotransposon insertion in intron.

1. 6 Regulation of floral induction in fruit trees such as apple by transgenic approaches

In the experiments in *Arabidopsis*, it was demonstrated that it is possible to accelerate or delay the flowering time by over-expressing or repressing genes related to flowering. We have started the studies on the apple genes related to flowering or juvenility since 1997 with the goal of producing the apples whose juvenile period is extremely reduced by regulating the expression of those genes. There are two ways to develop precocious flowering plants by genetic engineering. One is the constitutive expression of genes that promote flowering. Because the over-expression of *AP1*, *LEAFY* (*LFY*), or *FLOWERING LOCUS T* (*FT*), which are involved in

the switch from vegetative to reproductive phase, causes early flowering in *Arabidopsis*, the use of the flowering-promoting genes such as *API*, *LFY*, *FT* or their homologues will be effective also in producing precocious woody plants including apple. The other way is the suppression of genes that delay flowering. Because the loss-of-function *Arabidopsis* mutant of *TERMINAL FLOWER1* (*TFL1*) or *EMF2*, which represses the expression of *LFY* or *API*, respectively, in the wild-type plant, causes late flowering, the suppression of the flowering-repressing genes such as *TFL1* or *EMF2* homologues will be effective as well. Especially in the second way, the flowering-repressing genes originated from the host plants must be isolated and used for the vector construction to knockdown the endogenous gene by antisense expression, co-suppression, or RNA interference (RNAi).

In the production of precocious flowering woody plants by genetic manipulation, it was first reported that poplars (*populus* spp.) constitutively expressing *Arabidopsis LFY* flowered 7 months after regeneration. However, there was little success reported about the development of woody plants that showed precocious flowering except for the example of the transgenic poplars. These results suggested that the molecular and genetic mechanisms underlying floral induction were not always the same between herbaceous and woody plants. In woody plants, there might have been the mechanism that maintains juvenile phase more strictly than in herbaceous plants. Alternatively, key genes related to flowering or juvenility might be different between herbaceous and woody plants, although the fundamental mechanisms that underlie floral induction would be similar between them.

Thus, I made research plans to isolate flowering-promoting or repressing genes from apple. The flowering-promoting genes targeted were *LFY* and *API*, whereas the flowering-repressing gene targeted was *TFL1*. Isolation and expression analysis of apple homologues of *LFY* and *API* was described in the chapter 2. The morphological observation of flower development in apple was also described in this chapter. After the expression analysis of

those genes, the function analysis of *MdAP1* (an apple homologue of *AP1*) was performed using transgenic *Arabidopsis* ectopically expressing *MdAP1* as described in the chapter 3. Characterization of *MdTFL1* (an apple homologue of *TFL1*) was described in the chapter 4. Because *MdTFL1* was thought to be an apple orthologue of *TFL1* and involved in the maintenance of juvenile phase in apple, *MdTFL1* antisense gene was introduced into 'Orin' apple to suppress the expression of endogenous *MdTFL1*. The analysis of the transgenic apples with *MdTFL1* antisense gene was described in the chapter 5.

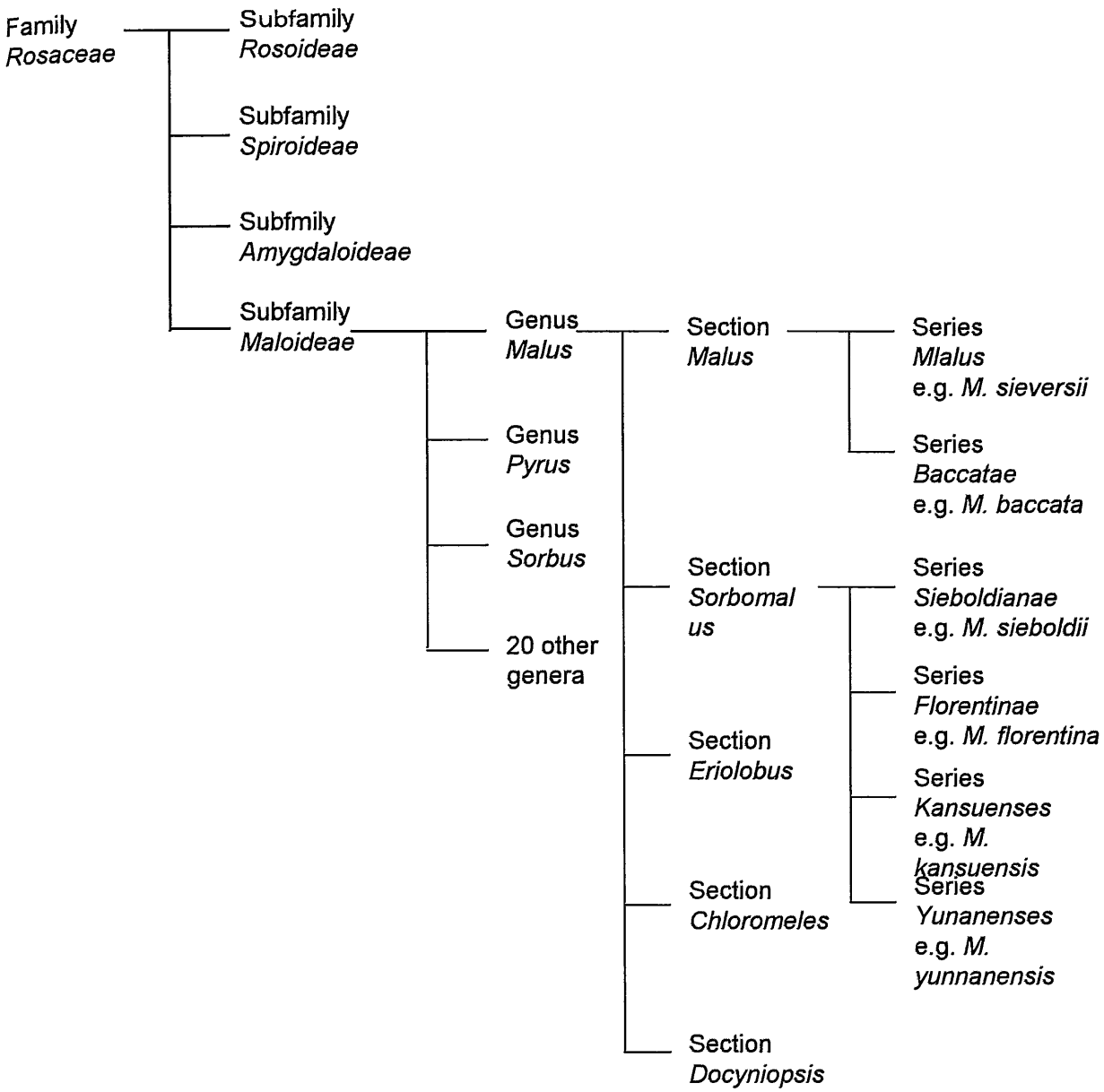

Fig. 1-1 Taxonomy of *Malus* (adapted from Phipps et al., 1991)

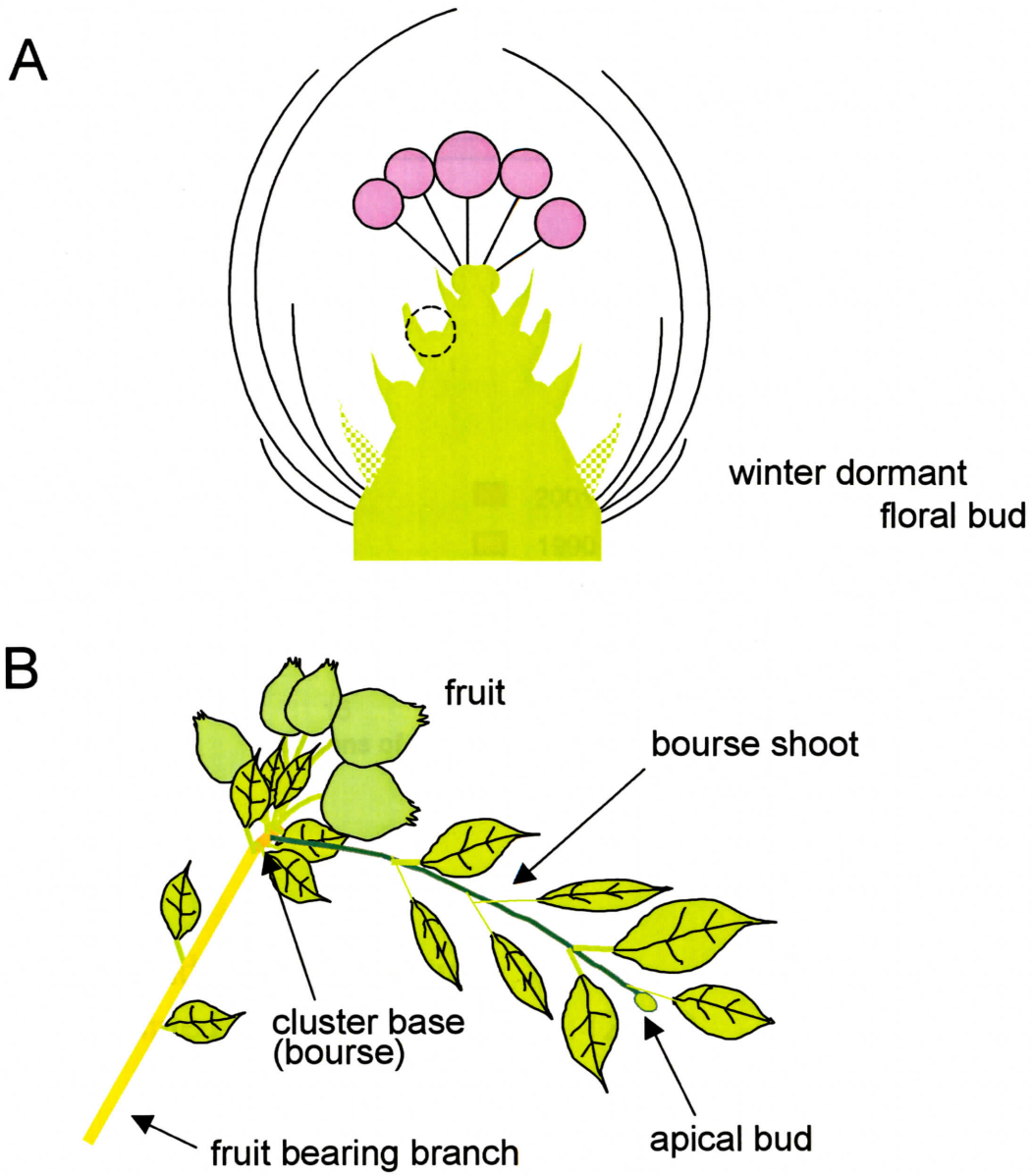

Fig. 1-2 Flowers developing from a bourse bud growing in the axils of leaves (adapted from Foster et al., 2003)

(A)Schematic representation of a winter dormant floral bud.
Curved lines, bud scales; hatched green parts, transition leaves; filled green parts, leaf promordia; pink circle, floral organs; dashed circle, bourse shoot.
(B) Schematic representation of a cluster base (bourse) and a bourse shoot.
Subsequent vegetative growth is from a bourse shoot that develops from the bourse.

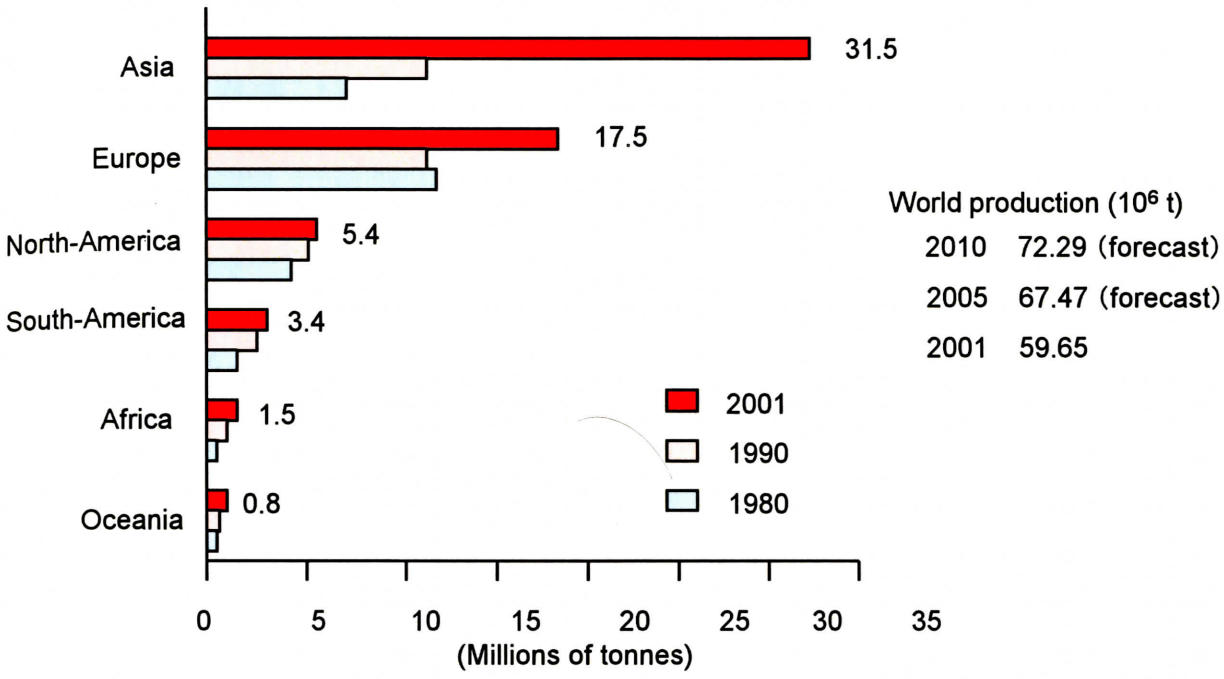

Fig. 1-3 World apple production
Adapted from O'Rourke et al. (2003)

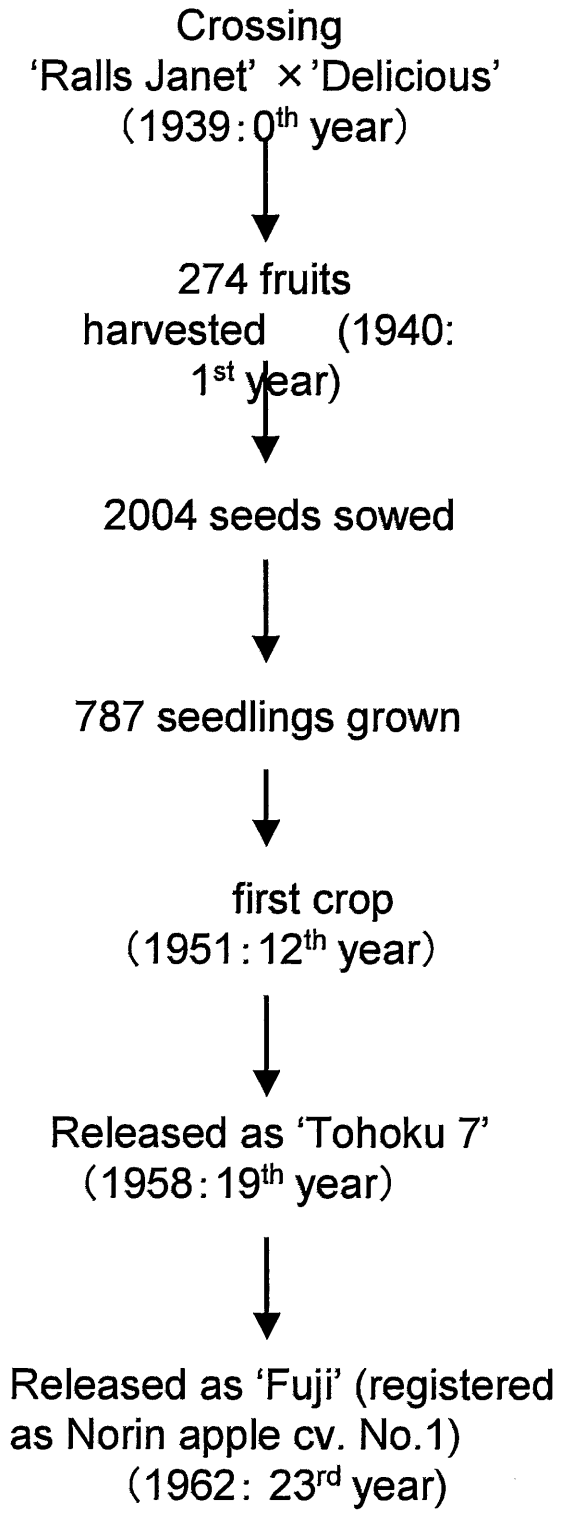

Fig. 1-4 Breeding progress of 'Fuji' apple

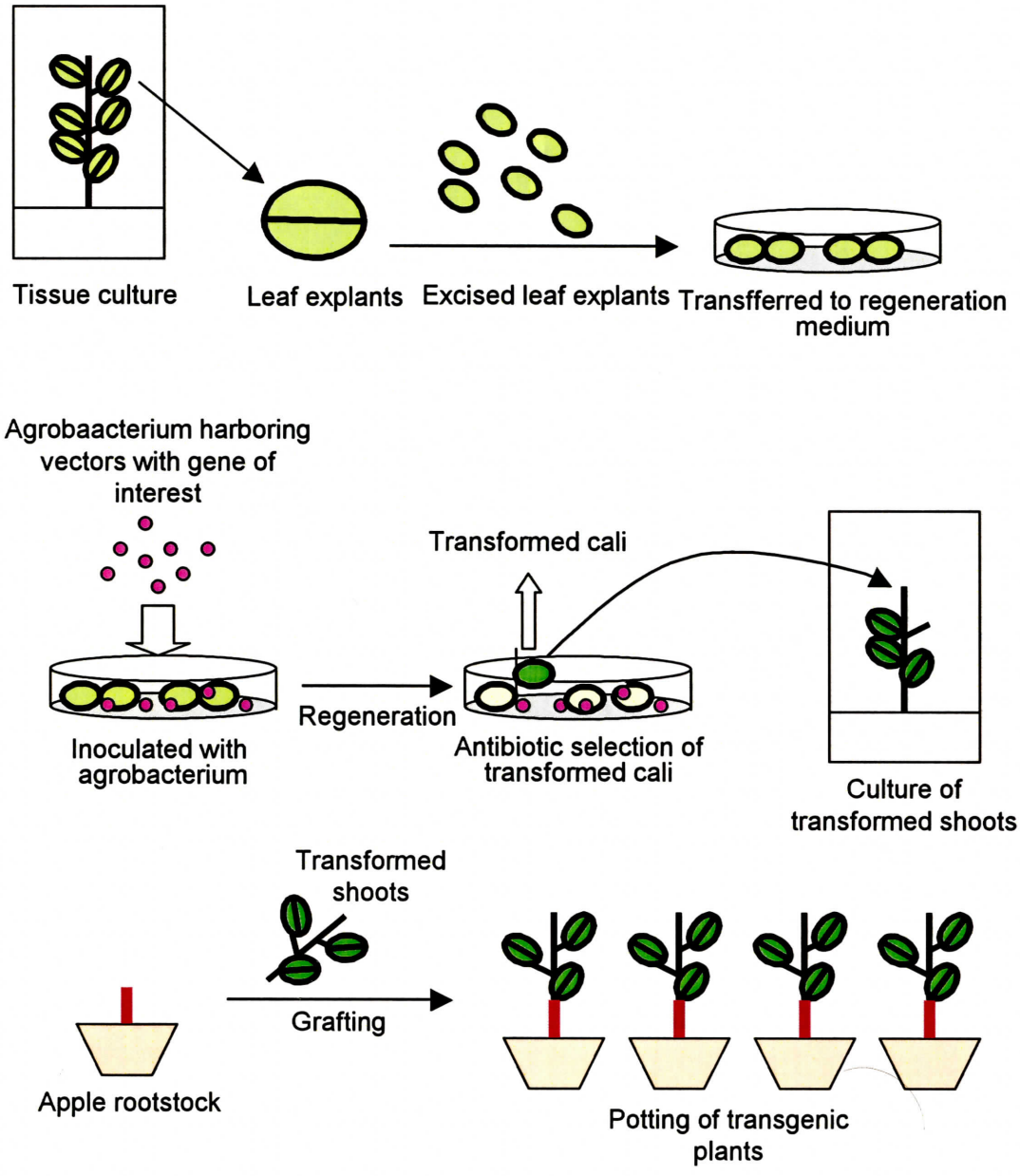

Fig. 1-5 The outline of genetic transformation of apple

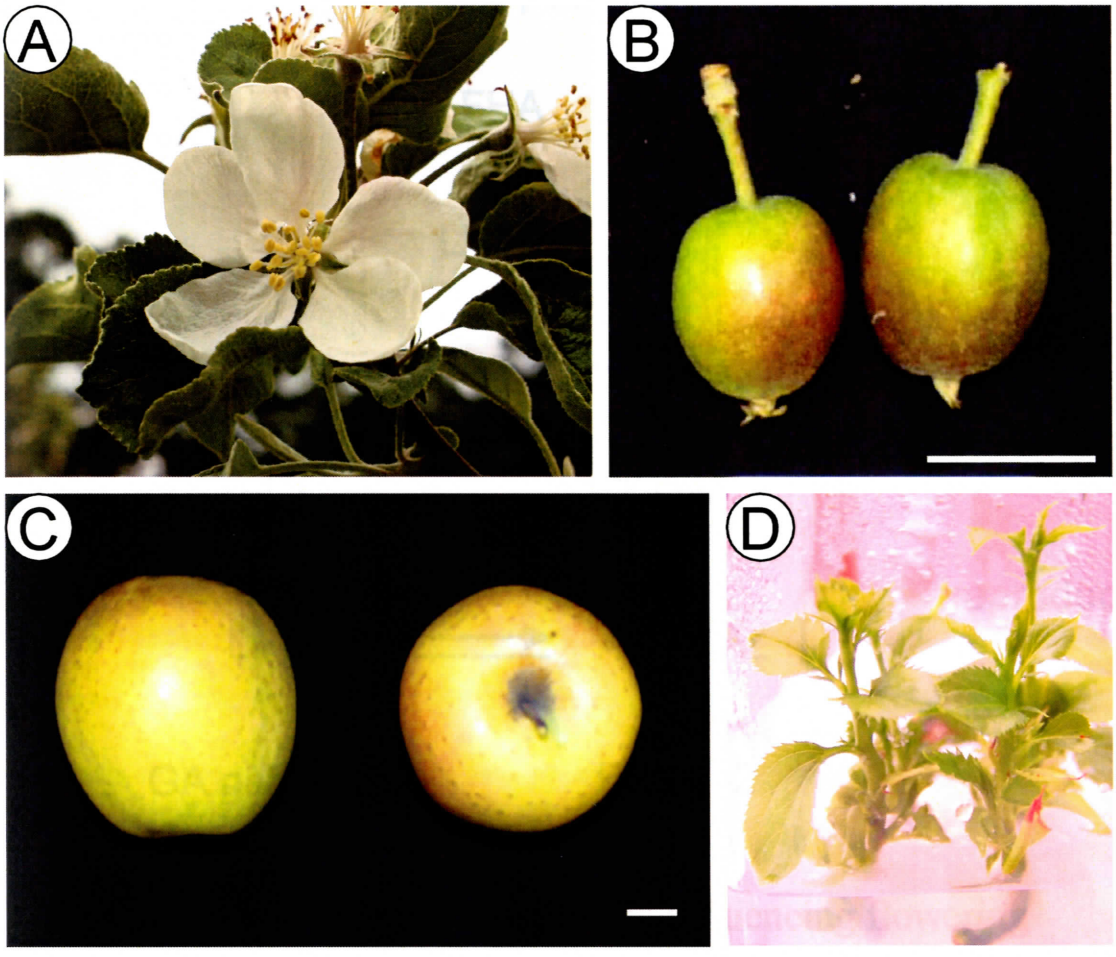

Fig. 1-6 Flower, fruit, and cultured tissue of 'Orin' apple

(A) Flower.
(B) Young fruit (5 WAFB).
(C) Mature fruit (22 WAFB).
(D) Cultured tissue.

Bars in (B) and (C) indicate 2 cm.

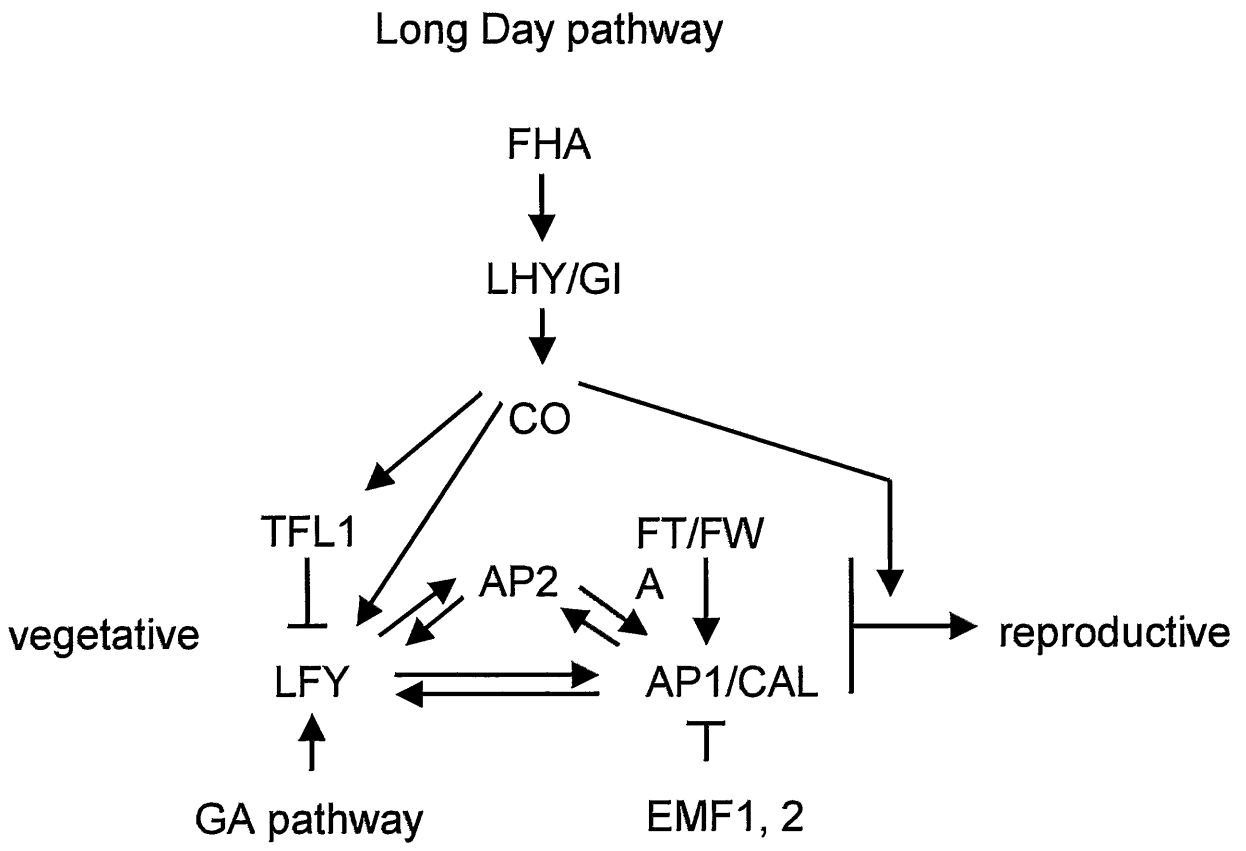

Fig. 1-7 The genetic and molecular interactions influencing flowering time and floral meristem identity in *Arabidopsis* (adapted from Piñeiro and Coupland,1998)

AP1 (*APETALA1*): Partial transformation of flowers into inflorescence in mutants.
AP2 (*APETALA2*): Partial transformation of flowers into inflorescence in mutants.
CAL (*CAULIFLOWER*): Partial transformation of flowers into inflorescence. in *ap1 cal* mutants.
CO (*CONSTANS*): Mutants flower late.
EMF1, 2 (*EMBRYONIC FLOWER1, 2*): Mutants flower early.
FHA (=*CRYPTOCHROME2*): Mutants flower mildly late.
FT (*FLOWERING LOCUS T*): Mutants flower late.
FWA (=*FTS*): Mutants flower late.
GI (*GIGANTEA*): Mutants flower late and have increased starch in leaves.
LFY (*LEAFY*): Mutants flower late.
LHY (*LATE ELONGATED HYPOCOTYL*): Mutants flower late.
TFL1 (*TERMINAL FLOWER1*): Mutants flower early.

Chapter 2

Isolation and expression analysis of apple genes *AFL* and *MdAP1*

2.1 Absrtract

Two apple (*Malus* x *domestica* Borkh.) 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 about 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.

2.2 Introduction

In contrast to herbaceous plants, apple (*Malus* x *domestica* Borkh.) trees 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; Piñeiro 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.

2.3 Materials and methods

2.3.1 Extraction of RNA from apple

RNA was isolated from 'Jonathan' apple [age: 14-15 yr; 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). Approximately 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℃ for 10 min. The solution was extracted with chloroform/isoamyl alcohol [24:1 (v/v)] and RNA in the aqueous phase was precipitated with 0.25 volume of 10 M lithium chloride at -20℃ for 2 h. Nucleic acids were pelleted by centrifugation at 12,000 g_n for 10 min at 4℃. 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 gn for 10 min at 4 ℃. The pellet was dissolved in TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA).

2.3.2 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 ℃. 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).

2.3.3 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'-CAG AGG GAG CAT CCG TTT ATC GTA AC-3' and 3' primer 5'-GAC GC/AA GCT TT/GG TT/GG GA/GA CAT ACC A-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 ℃, 1 min at 55 ℃, and 2 min at 72 ℃. 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 pre-mixed cycle sequence kit according to the manufacturer's instructions (Amersham, Buckinghamshire, United Kingdom) and universal primers in the Bluescript vectors on a Hitachi SQ5500 automated sequencer (Hitachi, Tokyo, Japan).

Four degenerate oligonucleotides (5' primers 5'-AAA/G GGI AAA/G T/CTI TTT/C GAA/G TA-3' and 5'-GAA/G CAA/G CAA/A T/CTI GAT/C ACI GC-3', 3' primers 5'-TTT/C TGT/C TCT/C TGA/G/T ATI GCT/C TT-3' and 5'-GCI GTA/G TCI AA/GT/C TGT/C TC-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 ℃, 1 min at 52 ℃, and 2 min at 72 ℃. 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.

2.3.4 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 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 65 ℃ 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'-CGT CAA GTC ACT TTT TGC AAA CGT-3' and 3' primer 5'-AGC ATC ACA GAG AAC AGA GAG C-3') were used to generate antisense DIG-labeled RNA probes.

2.3.5 in situ hybridaization

Probes for in situ hybridization were labeled with DIG-11-UTP using the DIG RNA labelling kit (Boehringer Mannheim). 1 μg of purified plasmid vector pBluescript II SK+ carrying 447 bp-long fragment of *AFL* cDNA was linearized with a restriction enzyme (EcoRI). Then, DIG-labeled RNA was synthesized in vitro using T7 polymerase.

Shoot apices at each developmental stage were fixed in FAA (1.8% formaldehyde, 5%

acetic acid, and 45% ethanol) for 24 h at 24 ℃. 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). The sections were mounted on the sillan coated glass slides. Slides were soaked sequentially in xylene-ethanol series (100% xylene for 10 min, 100% xylene for 5 min, xylene:ethanol (1:1 v/v) for 5 min × 2 times, 100% ethanol for 5 min × 2 times, to dissolve paraplast. After slides were dried completely for at least 1 h in vaccuum dessicator, they were soaked in ethanol series (100%, 90%, 70%, 50%, 30% ethanol for 2 min each and water for 5 min× 2 times) to be equilibrated with water. They were incubated in proteinase K solution [2-10 μg/ml proteinase K, 100 mM Tris, 50 mM EDTA (pH 7.5)] at 37℃for 30-40 min. They were washed in water for 5 min three times, then treated with acetic anhydride solution (0.1M triethanolamine and 0.5% acetic anhydride) at 25℃ for 10-15 min followed by two washes with 2 × SSPE (3 M NaCl, 173 mM NaH_2PO_4, 25 mM EDTA). Then, slides were dehydrated in the ethanol series (30%, 50%, 70%, 90%, 100% ethanol, 2 to 5 min each) and dried under vacuum for 1 h before hybridization. Slides were put in hybridization box, then hybridization mix (100-200 μl/slide) was applied and cover slips were covered. Hybridization was done at 50 ℃ in the dark for 16 h, followed by dipping in 4 × SSC at 25 ℃ for 15 min to fall down coverslips. Then coverslips being removed, slides were washed in 4 × SSC for 5 min three times, transferred to RNase buffer (5 M NaCl, 0.1 M Tris, 50 mM EDTA, pH7.5), then treated with RNaseA (50 μl/ml) in RNase buffer at 37℃ for 30min, followed by three washes in RNase buffer at 37℃ for 15 min and two washes in 0.5× SSC at 50℃ for 20 min. Immunological detection of the hybridized probe was carried out as described in the DIG RNA labelling kit (Boehringer Mannheim) with some modifications. Slides were washed with DIG buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH7.5) for 5 min two times, then incubated in 0.5% blocking reagent in DIG buffer 1 at 25 ℃ for 30 min followed by one rinse with DIG buffer 1. This was followed by a 2 h

incubation at 25℃in dilute antibody-conjugate (1:500 in 0.1% BSA and 0.2% Tween20 in DIG buffer1) and two washes of 15 min each. Slides were washed in DIG buffer3 for 5 min, and then put in wet box. Color solution (500-800 μl/slide) was applied and incubated in the dark for 6h-12h. After incubation, slides were put in TE (pH7.5) for 5 min, then washed in water for 5 min, followed by dehydration in ethanol series (30%, 50%, 70%, 90% ethanol, 2 min each and 100% ethanol, 5 min each). Finally slides were washed two times in xylene for 5 min, then mounted using entellan new (Merk Chemical).

2.4 Results

2.4.1 Morphological obserbation of flower development in apple

In apple, initiation of flower primordia for the following year occurs 3-6 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. 2-1). In 'Jonathan' apple, growth of most current shoots from the cluster base ceased in late June in Morioka, Japan (Fig. 2-2). From this observation, it appears the transition from the vegetative phase to the reproductive phase occurred in late June.

To determine the morphological changes of apical meristems during flower development, sections of apical buds were prepared (Figs. 2-3 and 2-4). Microscopic examination showed that the apical meristem was a narrow, pointed dome on 3 July (Fig. 2-3A), slightly domed on 17 July (Fig. 2-3B), and was clearly broadened by 31 July (Fig. 2-3C). 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. 2-3D), and sepal primordia were formed in early October (Figs. 2-3E and 2-4A). The initiation of stamen primordia occurred subsequently (Fig. 2-4A). In the middle of December, several flower primordia were visible (Fig. 2-3F). The floral organs expanded rapidly after the removal of dormancy and were fully differentiated in late April before bloom (Fig. 2-4B). In general, the inflorescence of apple consists of a terminal flower and four lateral flowers (Fig. 2-4A). The terminal flower usually grows and opens earlier than lateral flowers.

2.4.2 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. 2-5). 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. 2-6). 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. 2-5). The identity of *MdAP1* (*Malus* x *domestica AP1*) at the nuculeotide level was 68% with *SQUA* (Huijser et al., 1992) and

API (Mandel et al., 1992). At the amino acid level, the identity was 63% and 60% with *SQUA* and *API*, respectively (Fig. 2-6). 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 (Figs. 2-5 and 2-6) suggests 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' non-coding region.

2.4.3 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. 2-7A, 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. 2-7B). By contrast, *MdMADS* mRNA was detected at a very early stage as well as a late stage (Fig. 2-8). 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. 2-7A, 6.19V).

2.4.4 Expression of *AFL* and *MdAP1* in differnt tissues of apple

Expression patterns of *AFL* and *MdAP1* were analyzed in various 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. 2-9A). 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. 2-9B). 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).

2.4.5 in situ hybridization for *AFL* in apple apical buds

To demonstrate the spacial localization of *AFL* gene expression, in situ hybridization was performed at several develpmental stages of apple apical buds. On July 5th, when is before floral bud differentiation, no expression was detected (Fig. 2-10A). On July 12th, strong *AFL* expression was detected in the dome and leaf primordia (Fig. 2-10B). In the middle of July, flower bud differentiation starts in the apical buds of apple in reproductive phase as described in the section 2.4.1. After mid July, *AFL* expression was not detected again (Figure 2-10C, D) although *AFL* expression in apical buds was relatively strong in October and November from RNA blot analysis.

In the apical bud which is supposed to be a floral bud, *AFL* expression was detected

strongly on July 12th descrived above. *AFL* was expressed mainly in tunica layer and corpus, and slightly in rim meristem on shoot apex of apple (Fig. 2-11A). On the other hand, no *AFL* expression was detected in foliar bud on August 28th (Fig. 2-11B). This foliar bud was taken from current shoots called 'water sprout'. Usually, vegetative shoots like water sprout never differentiate floral buds nor come into flower.

2.5 Discussion

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 *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. 2-8). 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 about 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 about 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. × *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 with 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.

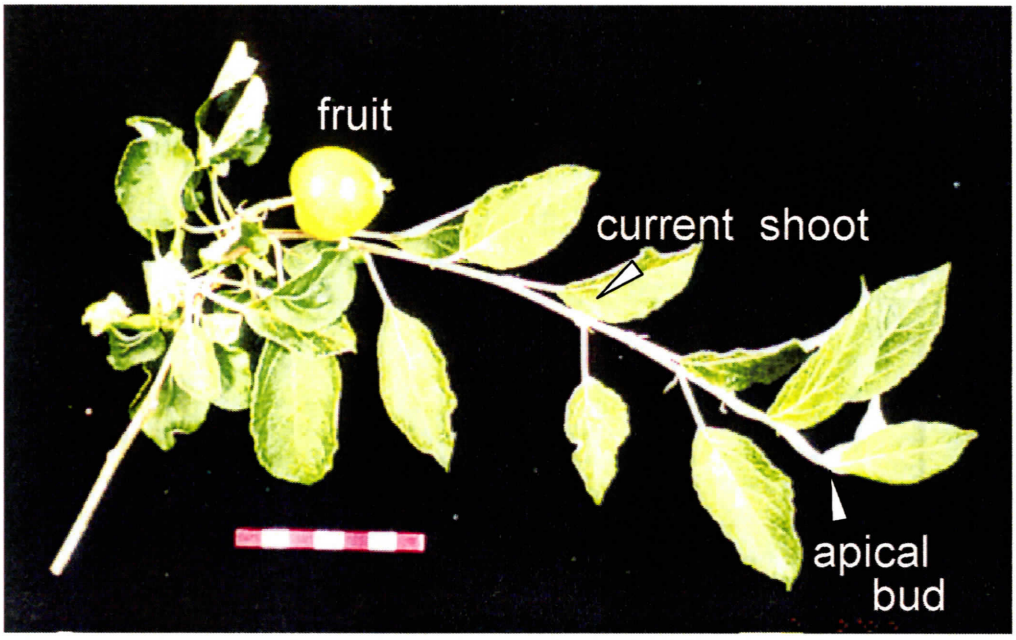

Fig. 2-1. Growth of current shoot (bourse shoot) of 'Jonathan' apple from the cluster base

Current shoots from the cluster base (bourse shoots) were collected for measuring shoot lengths and the total RNA was extracted from apical buds. Scale bar = 7 cm.

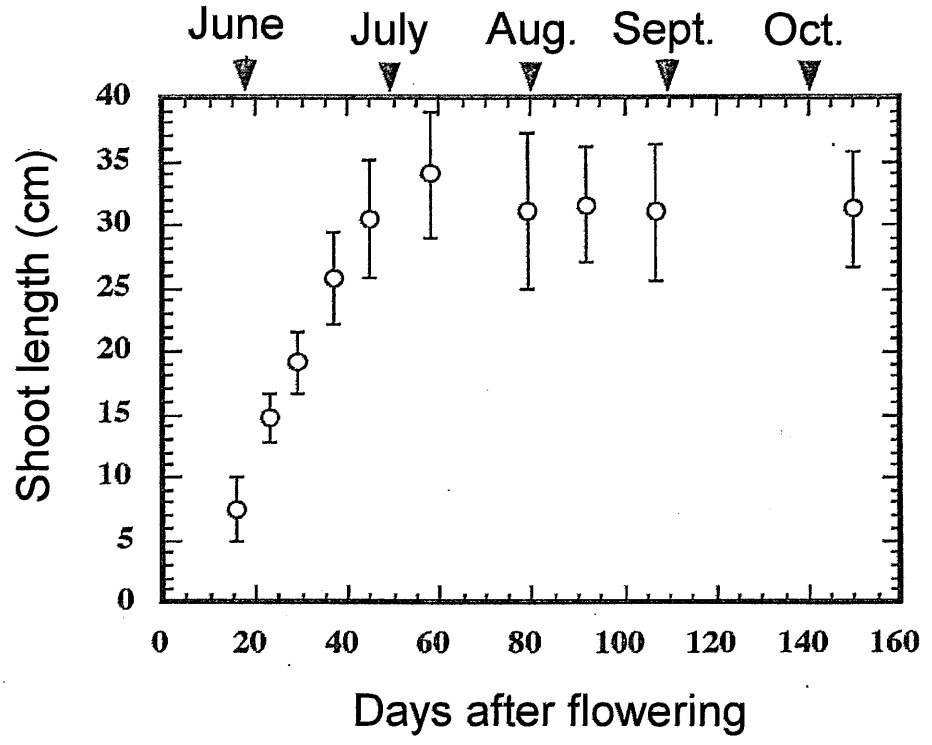

Fig. 2-2. The growth of current shoots during the floral bud differentiation

The lengths of 40-50 shoots were measured after flowering through October. Population of apical buds was synchronized at each developmental stage. Vertical bars = SD.

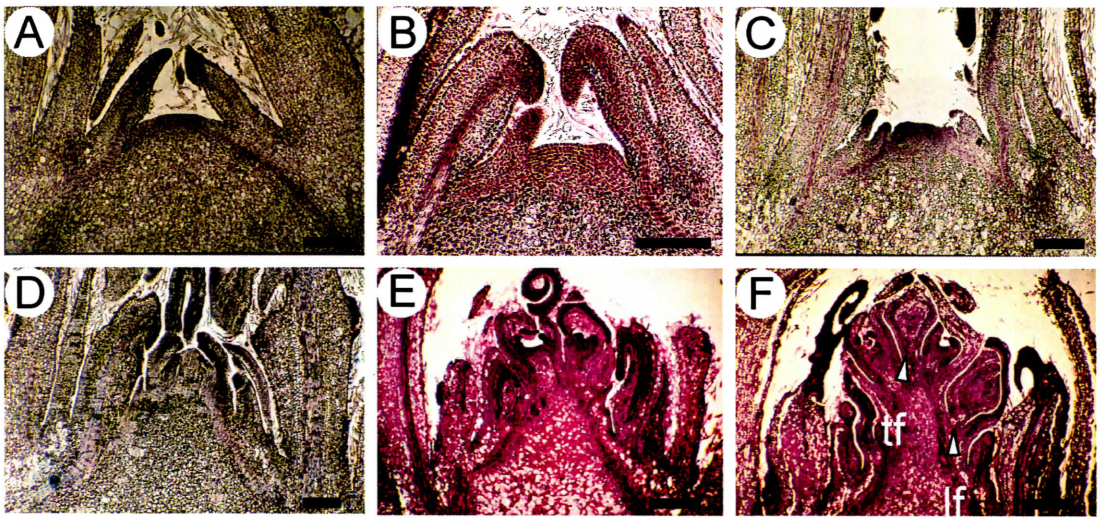

Fig. 2-3. Morphological changes of apical buds of 'Jonathan' apple

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, chromosomes, 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). Scale bars = 200 μm (A, B, C, D), 400 μm (E, F).

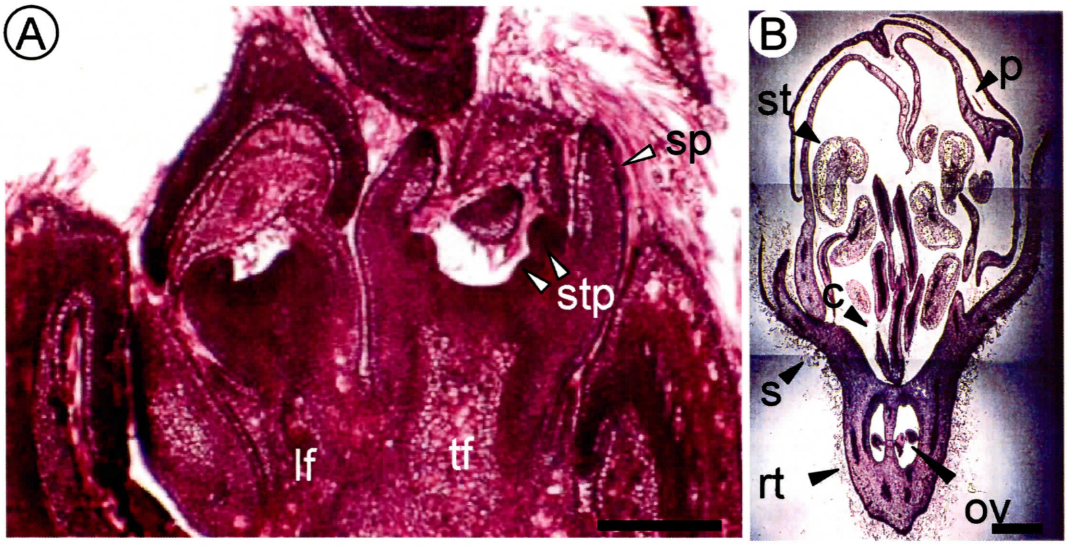

Fig. 2-4. Morphological changes of apical buds of 'Jonathan' apple at the late stage

(A) flower primordia on 10 Oct. (sp = sepal primordia, stp = stamen primordia, lf = lateral flower, and tf = terminal flower).
(B) 26 Apr. (p = petal, st = stamen, c = carpel, s = sepal, rt = receptacle, and ov = ovule). Scale bars = 1000 μm (A) and 200 μm (B).

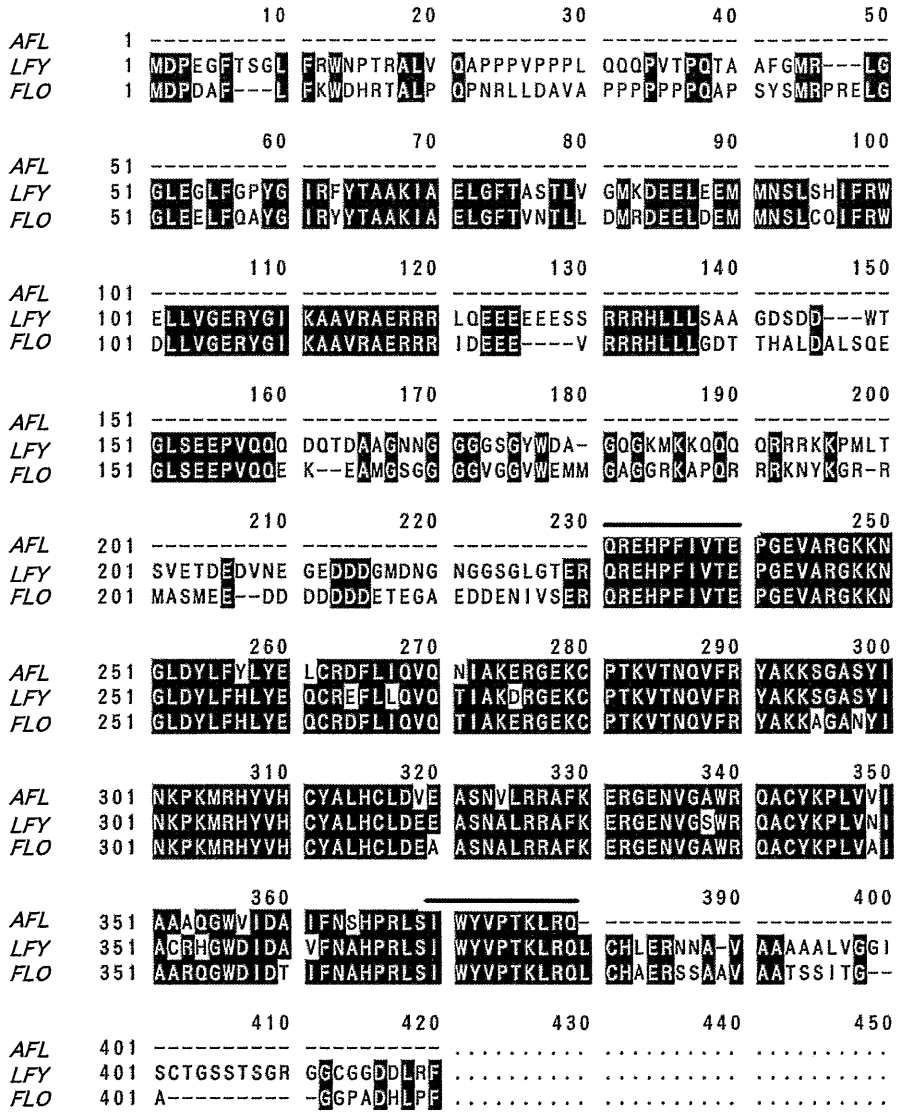

Fig. 2-5. Comparison of the *AFL* sequences with their homologues in *Arabidopsis* and *Antirrhinum*

Amino acids at positions of identity between two or three of these proteins are blocked in black. Black lines above *AFL* indicate primer sites for RT-PCR. The partial *AFL* obtained corresponded to 447 bp of the *FLO/LFY* coding sequence.

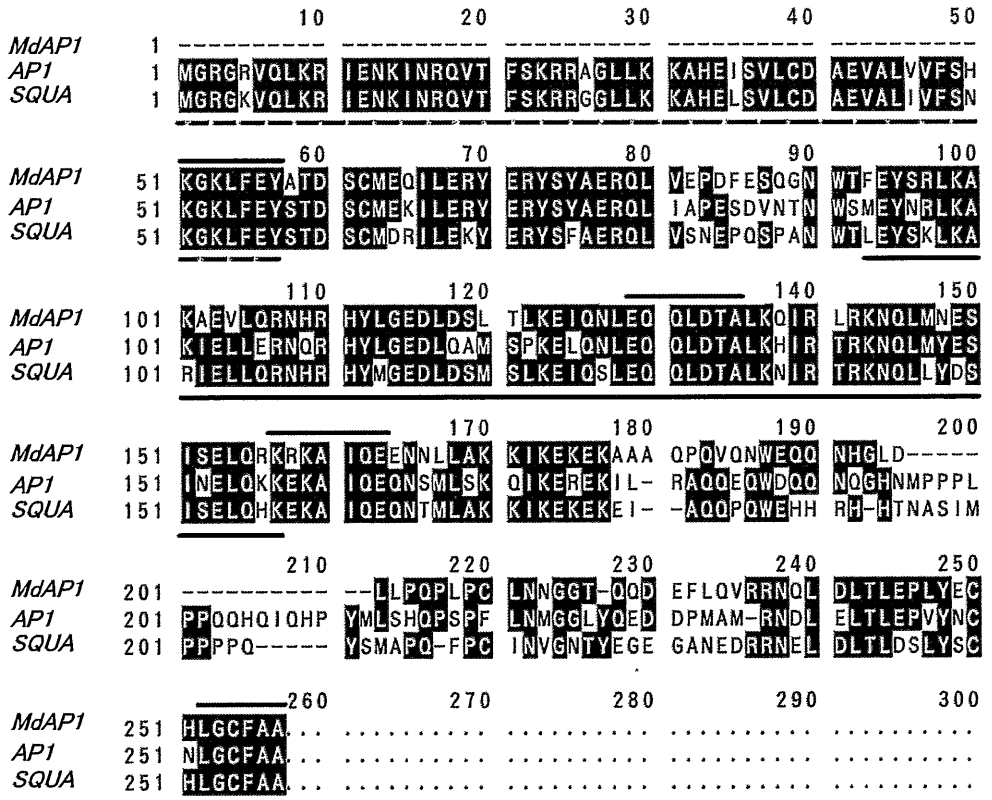

Fig. 2-6. Alignment of the partial *MdAP1* protein sequence with the *Arabidopsis AP1* and the *Antirrhinum SQUA* protein sequences

Amino acids at positions of identity between two or three of these proteins are blocked in black. Black lines above *MdAP1* indicate primer sites for RT-PCR. A broken line and a black line below the sequences indicate MADS-box and K-box regions, respectively.

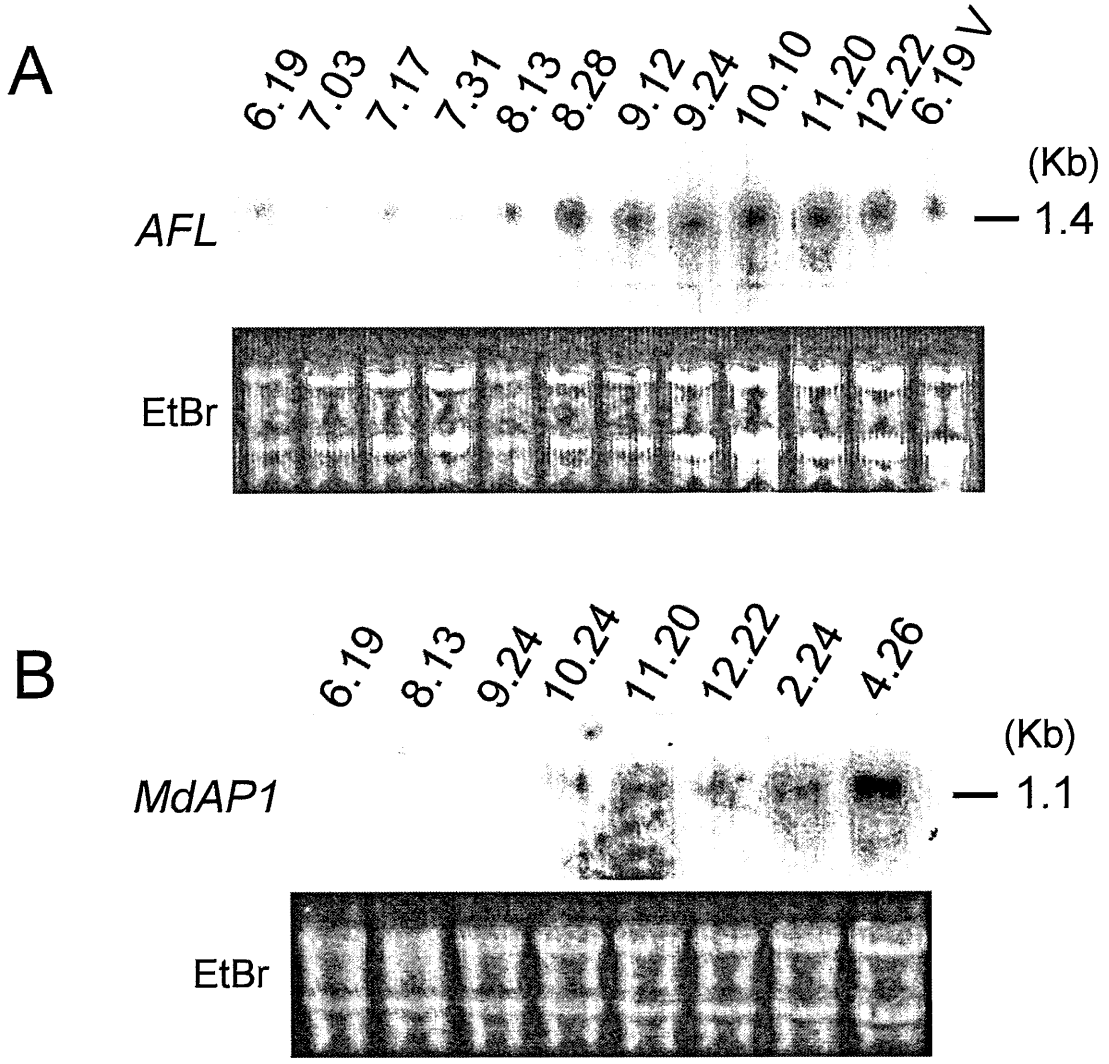

Fig. 2-7. Northern-hybridization expression pattern of *AFL* (A) and *MdAP1* (B) during flower development

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 was probed with DIG-labeled *AFL* or *MdAP1 fragments*.

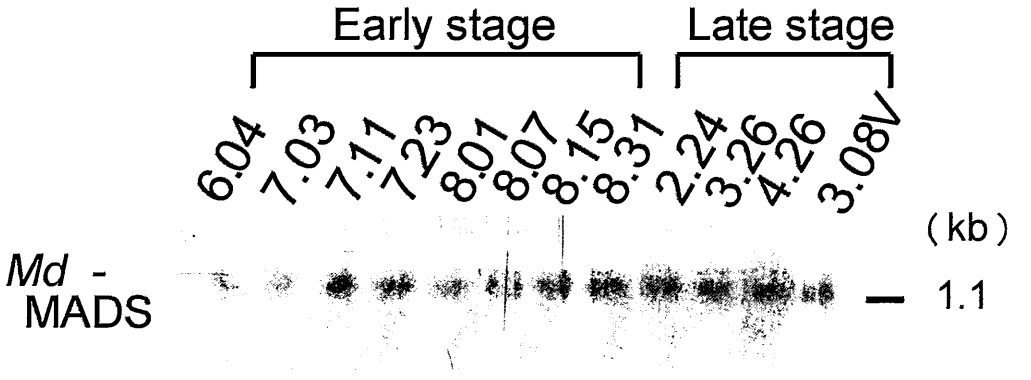

Fig. 2-8. Northern-hybridization expression pattern of *MdMADS* genes during flower development

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. V = vegetative shoot (water sprout).

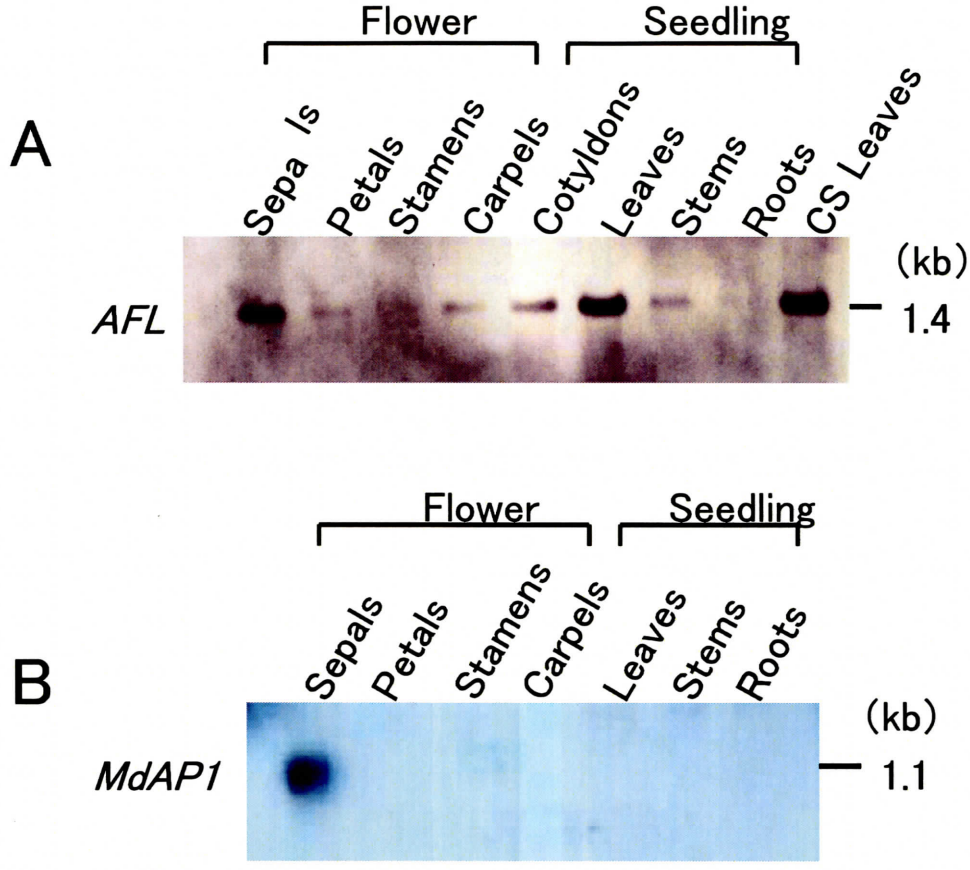

Fig. 2-9. Northern-hybridization expression pattern of *AFL* and *MdAP1* genes in different organs

(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 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).

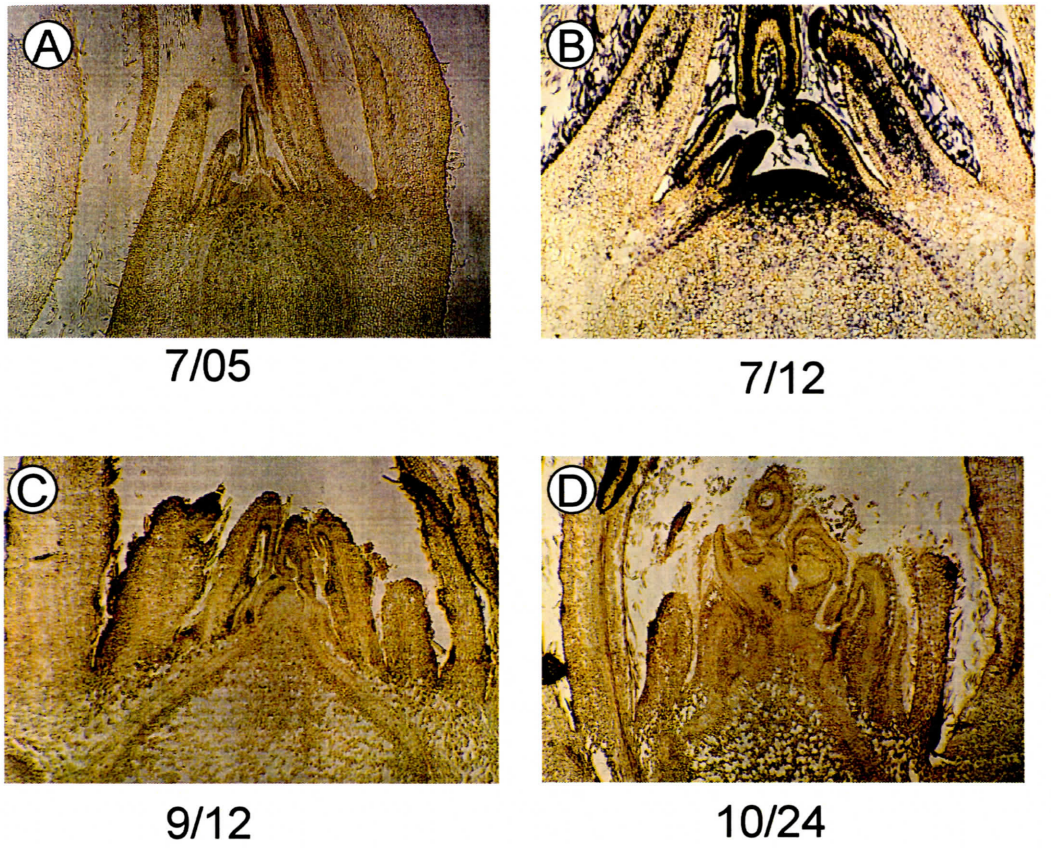

Fig. 2-10. *In situ* hybridization of longitudinal sections of floral meristems during the flower development in apple probed with DIG-labeled RNA probes for *AFL*.

Numbers below the photos indicate month/day.

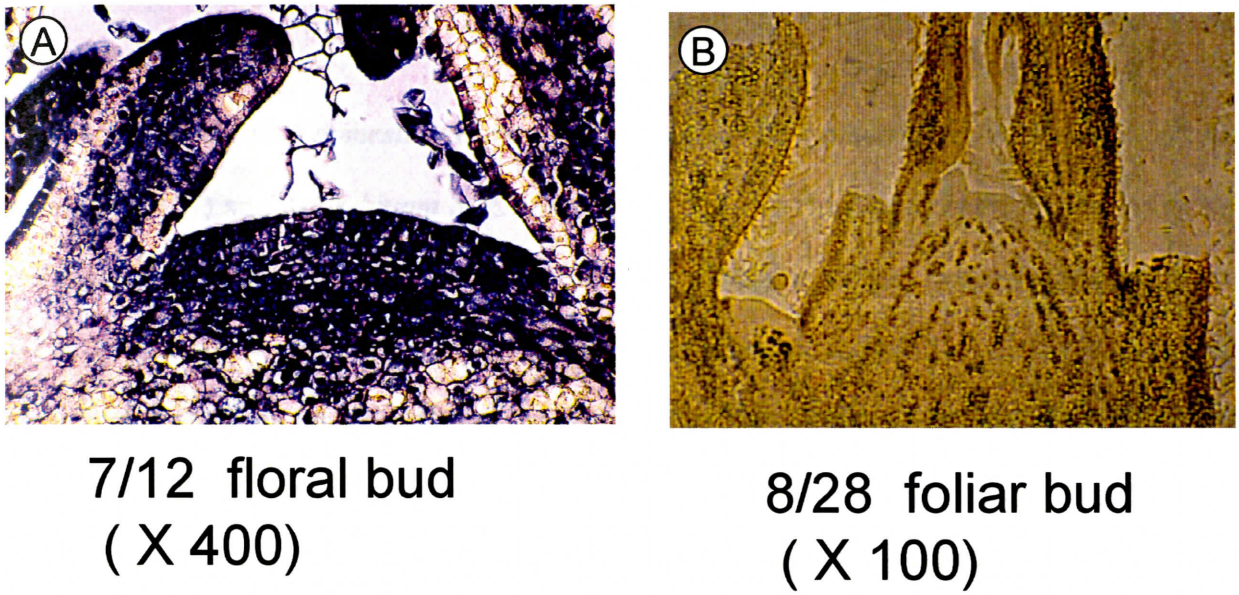

Fig. 2-11. *In situ* hybridization of longitudinal sections of floral meristems with DIG-labeled RNA probes for *AFL*.

Numbers below the photos indicate month/day.