

Developmentally regulated expression of two MADS-box genes, *MdMADS3* and *MdMADS4*, in the morphogenesis of flower buds and fruits in apple

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Abstract. Two MADS-box genes, *MdMADS3* and *MdMADS4*, were isolated from the apple (*Malus × domestica* Borkh.) cultivar Fuji, and their spatial and temporal expression patterns were studied during morphological differentiation of the flower buds and the fruits. Both *MdMADS3* and *MdMADS4* showed high sequence similarities to *FBP2* from petunia, *TM5* from tomato, and *AGL2*, *AGL4* from *Arabidopsis*. Although *MdMADS3* was expressed in the inner three whorls of the floral primordium, its expression was hardly detectable in developing fruit. The second gene, *MdMADS4*, was ubiquitously expressed in the inflorescence meristem, floral meristem, all four floral organs, and fruit. Moreover, *MdMADS4* expression was high in the vascular bundles assigned to the floral tube and the carpellary vascular bundles in fruit at early developmental stages. The *MdMADS4* transcript also accumulated in embryos of the developing seeds. These results suggest that *MdMADS3* and *MdMADS4* are involved in different functions, and that *MdMADS4* may function in the important events controlling flower and fruit development.

Key words: Flower and fruit development – MADS-box gene (*MdMADS3*, *MdMADS4*) – *Malus* (MADS-box gene) – Pome

Introduction

Genetic and molecular characterization of several floral homeotic loci in both *Arabidopsis thaliana* and *Antirrhinum majus* has indicated that MADS-box genes, which encode proteins sharing similarity with transcription factors from various eukaryotic organisms, direct flower

development. Plant MADS-box genes have a conserved DNA-binding domain called the MADS (MCM1, AΓAMOUS, DEFICIENS, and SRF) domain and a second conserved domain called the K domain that is involved in protein-protein interaction (Schwarz-Sommer et al. 1990; Ma et al. 1991; Davies et al. 1996). The phylogeny of the MADS-box gene family has revealed the existence of distinct gene subfamilies, which share highly related functions and expression patterns (Purugganan et al. 1995; Theißen et al. 1996). Most plant MADS-box genes play major roles during the development of the floral meristem or various organs (Yanofsky 1995; Amasino 1996; Levy and Dean 1998). Besides these groups with identified functions in flower development, many MADS-box genes were found that seem to have more-subtle functions, which are associated with floral meristem and organ identity (Pnueli et al. 1991; Argenent et al. 1992; Flanagan and Ma 1994; Savidge et al. 1995; Davies et al. 1996; Bonhomme et al. 1997; Mandel and Yanofsky 1998).

The apple belongs to the Maloideae subfamily of the Rosaceae, which is characterized by pome fruits (Rohrer et al. 1991). The Maloideae is an economically significant group of woody plants cultivated for their value as fruit crops, as well as for their ornamental beauty. The pome fruit is the most distinctive characteristic of the Maloideae subfamily (Rohrer et al. 1991, 1994). Regardless of recent advances in molecular biology, fewer advances have been made on the genetic control of temporal and spatial events during fruit set and fruit growth (Gillaspy et al. 1993). Moreover, information on the growth of pome fruits is restricted in scope since the majority of fruits are not pomes.

Recently, several MADS-box cDNA clones have been isolated from the flowers of fruit trees, including eucalypt and apple (Kyojuka et al. 1997; Sung and An 1997; Southerton et al. 1998; Sung et al. 1999; Yao et al. 1999). Analyses of the nucleotide sequences and expression patterns revealed that the fruit-tree genes share similarities with other plant MADS-box genes that function during floral development (Kyojuka et al. 1997; Southerton et al. 1998; Sung et al. 1999). It was

Abbreviation: RT-PCR = reverse transcriptase-polymerase chain reaction

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reported that some of the clones from eucalypt and apple were expressed in developing receptacles and fruits (Sung and An 1997; Southerton et al. 1998; Yao et al. 1999). However, the spatial and temporal expression patterns of the MADS-box genes during fruit development have not been studied. It is still unclear whether the MADS-box genes are involved in fruit set and growth of pome fruit. In the present study, we report characterization of the expression patterns of two MADS-box genes, which show high sequence relatedness to various MADS-box genes in the *AGL2* subfamily (Theißen et al. 1996). Possible regulatory roles of the apple MADS-box genes in flower and fruit development are discussed.

Materials and methods

Plant materials. The apple (*Malus × domestica* Borkh.) cultivar Fuji was used in this study. Plant samples were provided by the Kyungbuk Provincial Rural Development Administration (Taegu, Korea).

Isolation of *MdMADS* genes and construction of the phylogenetic tree. Copy-DNA clones were isolated from a cDNA library prepared from young flower buds according to Sung and An (1997). Overlapping subclones were created in a pBluescript SK(-) vector (Stratagene, La Jolla, Calif., USA), and nucleotide sequences were determined by cycle sequencing and running of the ABI 373A automatic sequencing system. Sequence analysis was carried out with the BLAST search and CLUSTALW programs. For the construction of phylogenetic trees, alignment of conceptual amino acid sequences was done by using the GCG program PILEUP. Phylogenetic trees were constructed as previously described (Münster et al. 1997).

Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. RNA was extracted from floral and fruit tissues as previously described (Sung and An 1997). First-strand cDNA synthesis was performed with 5 µg of total RNA (treated with RNase free DNase I) using the SuperScript pre-amplification kit (Life Technologies, Rockville, Md., USA) as directed by the manufacturer. A set of primers was designed to amplify a 251-bp DNA fragment of the *MdMADS3* transcript. The sense primer sequence was 5'-AGCTATCAGGACTATTTG-3' (I-region; nucleotides 349–366), and the anti-sense primer was 5'-TTCTTCCAGCTTCCTCTT-3' (C-region; nucleotides 583–600). For *MdMADS4*, a set of primers was designed to amplify a 447-bp DNA fragment of the *MdMADS4* transcript. The sense primer sequence was 5'-AGATACCAAGAATACTTG-3' (I-region; nucleotides 397–414), and the anti-sense primer was 5'-TCGAAATTCAGAGCATCC-3' (C-region; nucleotides 827–844). The PCR reaction was performed as previously described (Sung et al. 1998) with a slight modification as follows. The reaction mixture was denatured at 95 °C for 3 min, then incubated by a step program (94 °C, 30 s; 60 °C, 30 s) for 20 cycles afterwards. Fifteen microliters of the reaction mixture was separated on a 1.2% agarose gel, transferred, and hybridized with ³²P-labeled probe DNA using standard conditions.

In-situ RNA hybridization. Flower buds and fruits at different developmental stages were fixed, embedded, and sectioned as previously described (Sung et al. 1999). The 303-bp 3' region (nucleotides 646–949) of the *MdMADS3* cDNA and the 442-bp 3' region (nucleotides 549–991) of the *MdMADS4* cDNA were cloned into the pBluescript SK (-). These plasmids, designated pGA1528-3 and pGA1587-3, were used for synthesizing single-strand RNA probes of *MdMADS3* and *MdMADS4*, respectively. To generate

an antisense-RNA probe, pGA1528-3 was linearized with *EcoRI* and a single-strand RNA approximately 310 bp in length was synthesized by T7 RNA polymerase using the digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim, Germany). To generate an antisense-strand RNA probe for *MdMADS4*, the pGA1587-3 plasmid was linearized with *XhoI*, and a DIG-labeled RNA approximately 450 bp in length was synthesized using T3 polymerase. For the sense-strand RNA probe for *MdMADS4*, the pGA1587-3 plasmid was linearized with *EcoRI* and transcribed in vitro with T7 RNA polymerase. Hybridization conditions were same as previously described (Sung et al. 1999) except that hybridization temperatures were 43 °C and 50 °C for *MdMADS3* and *MdMADS4*, respectively.

Antibody preparation and protein immunolocalization. To express a truncated form of MdMADS3 and MdMADS4 proteins lacking the MADS domain, the 697-bp *EcoRI-HindIII* fragment of *MdMADS3* (amino acid residues 57–249) and the 684-bp *PstI-EcoRI* fragment of *MdMADS4* (amino acid residues 58–236) were cloned into the T7 expression vector, pRSET C (Invitrogen, Carlsbad, Calif., USA). These constructs were introduced into the *Escherichia coli* strain BL21 (pLysS), and the proteins were isolated, purified, and injected into rats as previously described (Sung et al. 1999). The antibodies were affinity-purified by using the western blot purification method (Burke et al. 1982). Plant tissues were prepared from young flowers, and the immunoreactive proteins were detected using a rat IgG ABC cassette kit (Vectastain Elite; Vector Laboratories, Burlingame, Calif., USA) and a substrate kit (VIP; Vector Laboratories). The stained sections were washed with distilled water for 5 min, then dehydrated and mounted in non-aqueous mounting media using standard protocols. Slides were examined on a microscope (Labophot-2, Nikon) using a bright field.

Results

Sequence analysis. Two different cDNA clones encoding MADS-box proteins, *MdMADS3* (accession No. U78949) and *MdMADS4* (accession No. U78950), were isolated from a cDNA library that was constructed from young flower buds of the apple cultivar Fuji. The *MdMADS3* cDNA clone is 1104 bp long and contains an open reading frame of 248 amino acid residues with a 81-bp 5' leader region and a 276-bp 3' untranslated region. The *MdMADS4* cDNA is 1055 bp long and contains an open reading frame of 235 amino acid residues with a 129-bp 5' leader region and a 218-bp 3' untranslated region. A multiple alignment of MdMADS3 and MdMADS4 with other MADS proteins (Fig. 1A,B) reveals that both MdMADS3 and MdMADS4 show high sequence relatedness to various MADS proteins in the *AGL2* subfamily (Theißen et al. 1996). The MdMADS3 protein shares 99% overall identity with MdMADS7, which was previously isolated from the apple cultivar Granny Smith (Yao et al. 1999); only one substitution is present at the 177th amino acid located in the C-region. The MdMADS4 protein shares the highest amino acid identity (63%) with CMB1 (accession No. L40404) of carnation. A phylogenetic analysis based on the amino acid sequences of the MIK region shows that both MdMADS3 and MdMADS4 belong to the *AGL2* subfamily (Fig. 2).

A		M
MdMADS4	2 GRGKVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIVFSTSGKLYEF	100 (%)
MdMADS3	2 ***R*****I**SR*****	92
MdMADS1	2 ***R*****I**NR*****	92
MdMADS7	2 ***R*****I**SR*****	92
MdMADS8	2 ***R*****I**NR*****	92
AGL2	2 ***R*****I**NR*****	92
AGL3	2 *****I**LI**NR*****	91
AGL4	2 ***R*****S*****NR*****	92
AGL9	2 ***R*****I**NR*****	92
CMB1	2 ***R*****NR*****	94
Egm3	2 *****I**NR*****	94
FBP2	2 ***R*****I**NR*****	92
PrMADS1	2 *****I**NR*****	94
TM5	2 ***R*****G*****I**NR*****	91

B		K	O
MdMADS4	91 YQEYLKLTKEVALQRTQRHLLGEDLVHLGTKEQLQLENQLDVSMKKIRSTKTQFMHVQISELQRKE	100	100 (%)
MdMADS3	91 **D**M**AR**V**QS**N*****S**N****EH**H**ET*L*Q***R****ILD*L*D**NR*	62	57
MdMADS1	92 *R**M**GRY*S*****N*****GP*N****E**R**EG*L*QV*****Y*LD*L*D**N**	65	59
MdMADS7	91 **D**M**AR**V**QS**N*****S**N****EH**H**ET*L*Q***R****ILD*L*D**NR*	62	57
MdMADS8	92 *R**M**GRY*S*****N*****GP*N****E**R**EG*L*QV*****Y*LD*L*D**N**	65	59
AGL2	92 *R*****GRY*N**Q**N*****GP*NS***E**R**G*L*QV**I***Y*LD*L*D**N**	64	56
AGL3	92 **D****SR**I**HS*****E*SEMDVN**EH**R*V*A*LRQ*****ARS*LD*L*D*KT**	55	57
AGL4	92 *R*****GRY*N**Q**N*****GP*NS***E**R**G*L*QV*CI***Y*LD*L*D**G**	62	56
AGL9	95 Q*****ERYD*****N*****GP*S****ES**R**S*L*Q**ALR****LD*LND**S**	64	52
CMB1	91 *****A**DV**SH*N*****GE*S****E**H**K*LRQ**I**H*LD*LAD**K**	65	63
Egm3	91 **D****AR**V**S**NPPW**E*GP*NS***E**H**EN*L*Q***A*****FD*LXH**H**	59	56
FBP2	93 Q*****ARY*****S**N*****GP*NS***ES**R**M*L*Q***R**L*LD*LQD*****	65	54
PrMADS1	91 **D**E**AR**V**S**N****E*GP*NS***E**H**EN*L*Q***A*****FD*LAH**H**	62	56
TM5	93 Q*****GRY*****S**N*****GP*NS***E**R**M*L*Q***R**L*LD*LTDY****	64	52

Fig. 1A,B. Comparison of the amino acid sequences of apple MADS proteins with other plant MADS proteins. **A** Sequence alignment of the MADS domains. Shown here are the deduced amino acid sequences of the MADS domains of the apple cultivar Fuji MdMADS4 (accession No. U78950), MdMADS3 (accession No. U78949), MdMADS1 (accession No. U78947), apple cultivar Granny Smith MdMADS7 (accession No. AJ000761), MdMADS8 (accession No. AJ001681), *Arabidopsis* AGL2 (accession No. P29382), AGL3 (accession No. P29383), AGL4 (accession No. P29384), AGL9 (accession No. O22456), carnation CMB1 (accession No. L40404), *eucalypt* Egm 3 (accession No. AF029977), petunia FBP2 (accession

No. M91666), Monterey pine PrMADS1 (accession No. U42399), and tomato TM5 (accession No. X60480). The *asterisks* represent amino acid residues identical to the corresponding ones in MdMADS4. The *numbers on the left* represent the positions of the first amino acid residues shown for each sequence. The *numbers on the right* represent the percentage of amino acid residues identical to the MADS domain (*M*) of MdMADS4. **B** Alignment of the K domains. The *asterisks* and the *numbers on the left* are as represented in **A**. The *numbers on the right* represent the percentage of identical amino acid residues with the K domain (*K*) and the overall region (*O*) of MdMADS4

Assay by RT-PCR. The deduced amino acid sequence of *MdMADS3* differed from that of *MdMADS7* in only the one position located in the C-region. It has been reported by RNA blot analysis that *MdMADS7* is

highly expressed in apple fruit (Yao et al. 1999). However, we have experienced cross-hybridization of the *MdMADS3* probe with other MADS-box genes in apple even though the highly conserved MADS-box

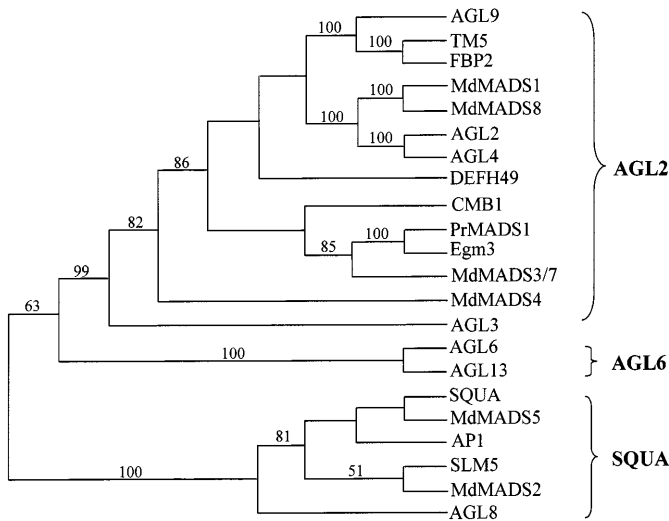


Fig. 2. Phylogenetic tree of the MIK domains of apple and several dicot MADS-box genes. Numbers next to the nodes indicate bootstrap values from 100 replicates. Shown here are *Arabidopsis* AP1 (accession No. P35631), AGL6 (accession No. M55554), AGL8 (accession No. U33473), AGL13 (accession No. U20183), snapdragon DEF49 (accession No. X95467), SQUA (accession No. X63701), white campion SLM5 (accession No. X80492), apple cultivar Fuji MdMADS2 (accession No. U78948), apple cultivar Granny Smith MdMADS5 (accession No. AJ000759), and the MADS-box proteins described in Fig. 1

region was not included in the probe (data not shown). Therefore, the RT-PCR approach was performed to increase the specificity.

The results from the RT-PCR analysis are shown in Fig. 3. In a comparison of the levels of transcript accumulation in flower buds and fruit following pollination, *MdMADS3* expression was found to be similar in buds and fruit whereas the *MdMADS4* signal was detected at a higher level in RNA from fruit. In fully expanded leaves, both *MdMADS3* and *MdMADS4* transcripts were undetectable.

Expression patterns of *MdMADS3* and *MdMADS4* during the early period of flower development. The apple has a determinate inflorescence with a terminal flower and a tendency toward dichasial branching (Pratt 1988). The differentiation of flower buds is initiated in July in

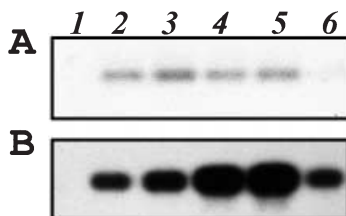


Fig. 3A,B. Analysis by RT-PCR of *MdMADS3* (A) and *MdMADS4* (B) in leaves, flower buds, and fruit of apple following pollination. Lanes: 1, fully expanded leaves; 2, flower buds (bud length = 5–6 mm); 3, post-anthesis flower; 4, fruit (diameter = 2.5 mm); 5, fleshy tissue of the fruit (diameter = 3.5 mm); 6, seeds of the fruit (diameter = 3.5 mm)

Korean climates, and the differentiation proceeds until dormancy occurs in winter. An early stage of flower bud development can be divided into three stages; evocation of the inflorescence meristem followed by the floral meristem (stage 1), differentiation into flower primordia (stage 2), and sequential initiation of sepal, petal, stamen, and carpel primordia (stage 3).

Expression of *MdMADS3* was first detected at stage 3 in the inner three whorls of the floral primordium (Fig. 4D). No *MdMADS3* RNA was detectable either in younger flower primordia or in the inflorescence meristem (Fig. 4A–C). In sections probed with the *MdMADS4* antisense RNA, a strong signal of the *MdMADS4* transcript was seen in the inflorescence meristem and the tips of the subtending leaf appendages during stage 1 (Fig. 4E). Apples have a determinate inflorescence with a terminal flower; the flower meristem emerges on the apex of the inflorescence meristem. At late stage 1, *MdMADS4* was highly expressed in the floral meristem that began to emerge from the inflorescence meristem and the tips of the subtending leaf appendages (Fig. 4F). A weak signal was observed in the bud procambium. When the floral meristem differentiated into six flower primordia (stage 2), the *MdMADS4* signal was high in the floral meristem region that gives rise to petal, stamen, and carpel primordia (Fig. 4G). A lower expression of the *MdMADS4* gene was found in the developing sepal primordia. At stage 3, *MdMADS4* was expressed in all four floral organs (Fig. 4H). The sense probe of *MdMADS4* did not show any appreciable hybridization signal (Fig. 4I–L) in the flower bud sections from stages 1 to 3. At the end of stage 3, apple trees entered a dormant period.

Expression patterns of *MdMADS3* and *MdMADS4* during the late period of flower development. In the spring, the flower buds resume growth. The flowers are completed between bud burst and anthesis. In apples, the critical stage of flower development is the period when the anthers and pistil primordia appear, and the beginning of differentiation of the pollen mother cell (Buban and Faust 1982). At this stage (stage 4), the *MdMADS3* signal clearly declined in petals, anthers, and fused carpels (Fig. 5A). Whereas, *MdMADS4* expression was still high in all four floral organs, with the signal being particularly strong in anthers and fused carpels (Fig. 5D). As the flowers matured, *MdMADS3* expression was no longer detectable in the flower (Fig. 5B), but *MdMADS4* transcript was detected at high levels in the ovules and the floral tube, which is the fused bases of sepals, petals, and stamens (Fig. 5E). Some of the flower primordia initiated in the previous growing season never reached full development, with leaflet pistils and insufficiently developed anthers (Pratt 1988). As shown in Fig. 5C and F, neither *MdMADS3* nor *MdMADS4* was expressed in this epigenetic aberrant flower, suggesting that both MADS genes are involved in full development of flowers.

Expression of the protein product of *MdMADS4* in early flower development. Some MADS-box genes, such as the

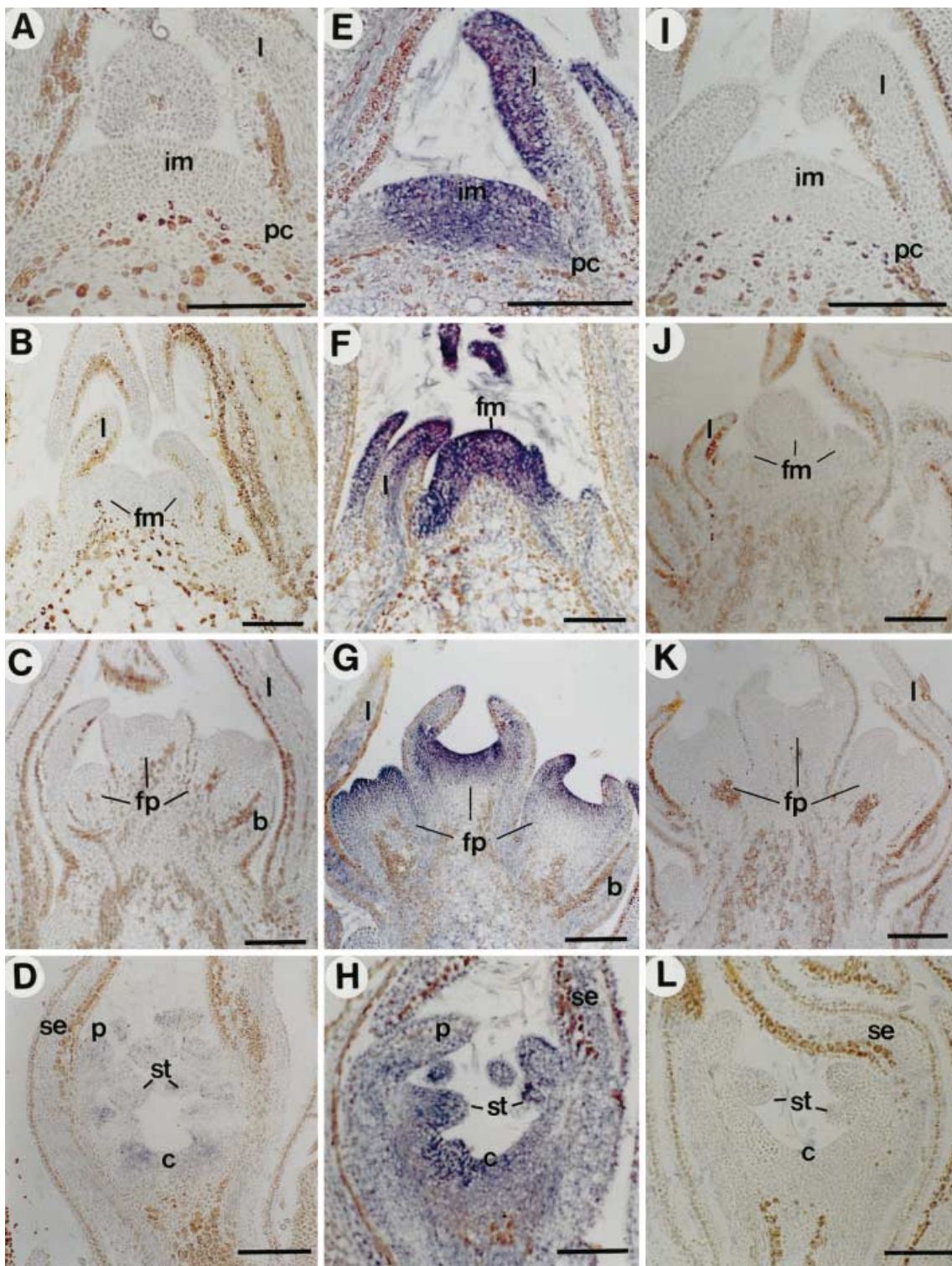
*MdMADS3**MdMADS4*

Fig. 4A–L. Patterns of *MdMADS3* and *MdMADS4* RNA expression during the early stages of apple flower development. Longitudinal sections of flower buds were hybridized with antisense-RNA of *MdMADS3* (A–D), antisense-RNA of *MdMADS4* (E–H), and sense-RNA of *MdMADS4* (I–L). The transcript signal is blue. Bars = 150 μ m. A,E,I Stage 1 flower buds with the inflorescence meristem. B,F,J Stage 1 flower buds with the floral meristem arising

on the apex of the inflorescence meristem. C,G,K Stage 2 flower buds with young flower primordia. D,H,L Stage 3 flower buds with developing floral organ primordia. b, bract; c, carpel primordium; fm, floral meristem; fp, flower primordium; im, inflorescence meristem; l, leaf appendage; p, petal primordium; pc, procambium; se, sepal primordium; st, stamen primordium

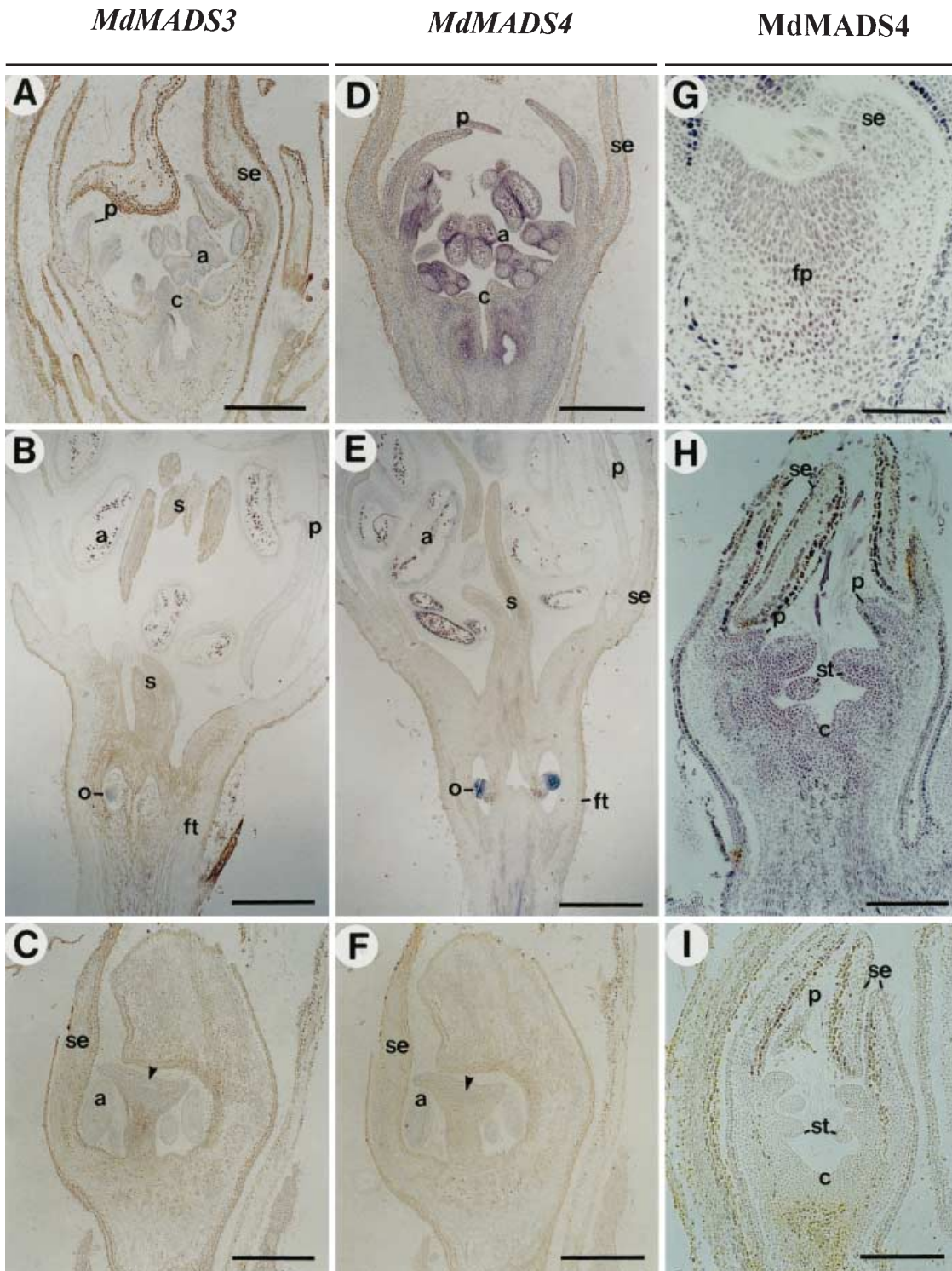


Fig. 5A–I. Patterns of *MdMADS3* and *MdMADS4* RNA expression during the late stages of apple flower development, and expression patterns of *MdMADS4* protein in the stages of floral organ initiation. Longitudinal sections of flower buds were hybridized with antisense-RNA of *MdMADS3* (A–C), antisense-RNA of *MdMADS4* (D–F), and affinity purified antibodies of *MdMADS4* (G–H). The transcript signal is blue and the protein signal is purple. **A,D** Stage 4 flower buds with the developing anthers and fused carpels. Bars = 500 μ m. **B,E**

Mature flowers at pre-anthesis stage. Bars = 1 mm. **C,F** Epigenetic aberrant flower buds with leaflet pistils and immature anthers. The *arrowheads* indicate the leaflet pistils. Bars = 500 μ m. **G** Stage 2 flower bud with young flower primordia. Bar = 100 μ m. **H** Stage 3 flower bud with the four floral organ primordia. Bar = 200 μ m. **I** Preimmune serum control for **H**. Bar = 200 μ m. *a*, anther; *c*, carpel; *fp*, flower primordium; *ft*, floral tube; *o*, ovule; *p*, petal; *s*, style; *se*, sepal; *st*, stamen

petunia floral binding protein (*fbp1*) gene, *Arabidopsis* *AP3* gene, and apple *MdMADS2* gene, are subjected to posttranscriptional regulation during morphological differentiation of the flower (Canas et al. 1994; Jack et al. 1994; Sung et al. 1999). We have previously demonstrated that the *MdMADS2* gene is a member of the *SQUA* subfamily, and that the expression of MdMADS2 protein was excluded from the stamen and carpel primordia, in which a considerable *MdMADS2* mRNA signal was detected (Sung et al. 1999). In order to investigate whether the *MdMADS3* and *MdMADS4* genes are also controlled posttranscriptionally, antibodies were generated against the MADS proteins. We have observed that the MdMADS4 antibodies were specific for detection of its own protein, whereas the MdMADS3 antibodies cross-hybridized to other MADS-box proteins (data not shown).

We found that the expression patterns of the MdMADS4 protein match those of the *MdMADS4* mRNA in the early stages of flower development (Fig. 5G–H). The MdMADS4 protein accumulated in the floral meristem region that gives rise to petal, stamen, and carpel primordia, and at a lower level in the sepal primordia (Fig. 5G). When differentiation of the four floral organ primordia became apparent, MdMADS4 continued to be expressed in the primordia of petals, stamens, and carpels, and in the developing sepals (Fig. 5H). Relatively strong signals of MdMADS4 were seen in the epidermal cells of the developing sepals, and in the inner three whorls of the floral primordium. Expression of MdMADS4 was also evident in the rib vascular bundle which is very active in the formation of the elongated receptacle. No staining was detected in control experiments utilizing preimmune serum as the primary antibody (Fig. 5I).

Expression patterns of MdMADS3 and MdMADS4 during fruit development. When the flowers open, anthesis and fertilization takes place. The floral tube surrounding the ovary swells and makes up the bulk of the fruit's fleshy tissue. The overall hybridization signal of *MdMADS3* RNA was undetectable in the fruits (Fig. 6A–D). Absences of detectable *MdMADS3* transcript in in-situ experiments in fruits conflicted with RT-PCR experiments, which showed the presence of *MdMADS3* transcript in fruits. This may be due to elongation of the cells and the enlargement of their vacuoles. In contrast to *MdMADS3*, the *MdMADS4* transcript was abundantly expressed along the vascular bundles in the floral tube and in the region interior to the integument in ovules at one week after anthesis (Fig. 6E,I). Two weeks after anthesis, *MdMADS4* expression was continued in the ovules and the vascular bundles located along the entire length of floral tube (Fig. 6F). In cross-sectional view (Fig. 6J), *MdMADS4* was highly expressed in the fertilized ovules. A strong expression of *MdMADS4* was also observed in the vascular bundles assigned to the floral tube, which include petal vascular bundles, sepal vascular bundles, and their outwardly directed branches. A lower expression was detected in the carpellary vascular bundles. The level of *MdMADS4* expression

became weaker as fruit growth proceeded (Fig. 6G). During seed development, *MdMADS4* was expressed in developing embryos (Fig. 6H).

Discussion

Two MADS-box genes that show high sequence relatedness to members of the *AGL2* subfamily were isolated from the Fuji apple. Genes in the *AGL2* subfamily show relatively heterogeneous expression patterns, suggesting that they are not functionally a homogeneous class of genes (Theißen et al. 1996). The *MdMADS3* and *MdMADS4* showed different expression patterns during morphological differentiation of the flower buds. While *MdMADS3* was expressed in the petal, stamen, and carpel primordia in stage 3 flower buds, it was not expressed either in younger flower primordia or the inflorescence meristem. In contrast to *MdMADS3*, *MdMADS4* was expressed both in the inflorescence meristem and the floral meristem, and it was ubiquitously expressed in all four whorls of the flower.

The expression pattern of *MdMADS3* is similar to those of *FBP2* from petunia, *TM5* from tomato, and *AGL9* from *Arabidopsis*, which are expressed in petals, carpels, and stamens (Angenent et al. 1992; Pnueli et al. 1994; Mandel and Yanofsky 1998). The genes *FBP2*, *TM5*, and *AGL9* are expressed after the onset of the meristem-identity genes, but before the activation of organ-identity genes, suggesting a possible role as mediators between the floral meristem and floral organ-identity genes. The *egm1* and *egm3* genes from eucalypt and the *DEFH49* gene from *Antirrhinum majus* are also expressed in the inner three whorls of the flower (Davies et al. 1996; Southerton et al. 1998). The expression pattern of *MdMADS4* is more similar to that of *AGL2*, which is expressed in the floral meristem and all four whorls of the *Arabidopsis* flower (Flanagan and Ma 1994). The *AGL3* gene from *Arabidopsis* is expressed in leaves and stems, as well as in flowers (Huang et al. 1995). However, neither *MdMADS3* nor *MdMADS4* were expressed in leaves.

Flowers of the apple develop into pome fruits, which is the most distinctive characteristic of the Rosaceae family. In nearly all members of the Rosaceae, a conspicuous feature of the flower is the floral tube, cup-like receptacle, or hypanthium (Rohrer et al. 1991). The exact morphological origin and nature of the floral tube has long been debated, mostly through investigations of the fruits of *Malus* and *Pyrus*. According to the "receptacular" theory, the receptacle forms the cup, so morphologically the floral tube is a stem tissue (Pratt 1988; Rohrer et al. 1991). The "appendicular" theory holds that the floral tube consists of adnated bases of the sepals, petals, and stamens, and thus the floral tube is morphologically a leaf tissue (Pratt 1988; Rohrer et al. 1991). Apple fruits are commonly described as having carpellary tissue (core) and a floral tube (flesh outside of the core line), terms which imply the appendicular theory. We have examined the morphological features during fruit set and growth using the *MdMADS3* and

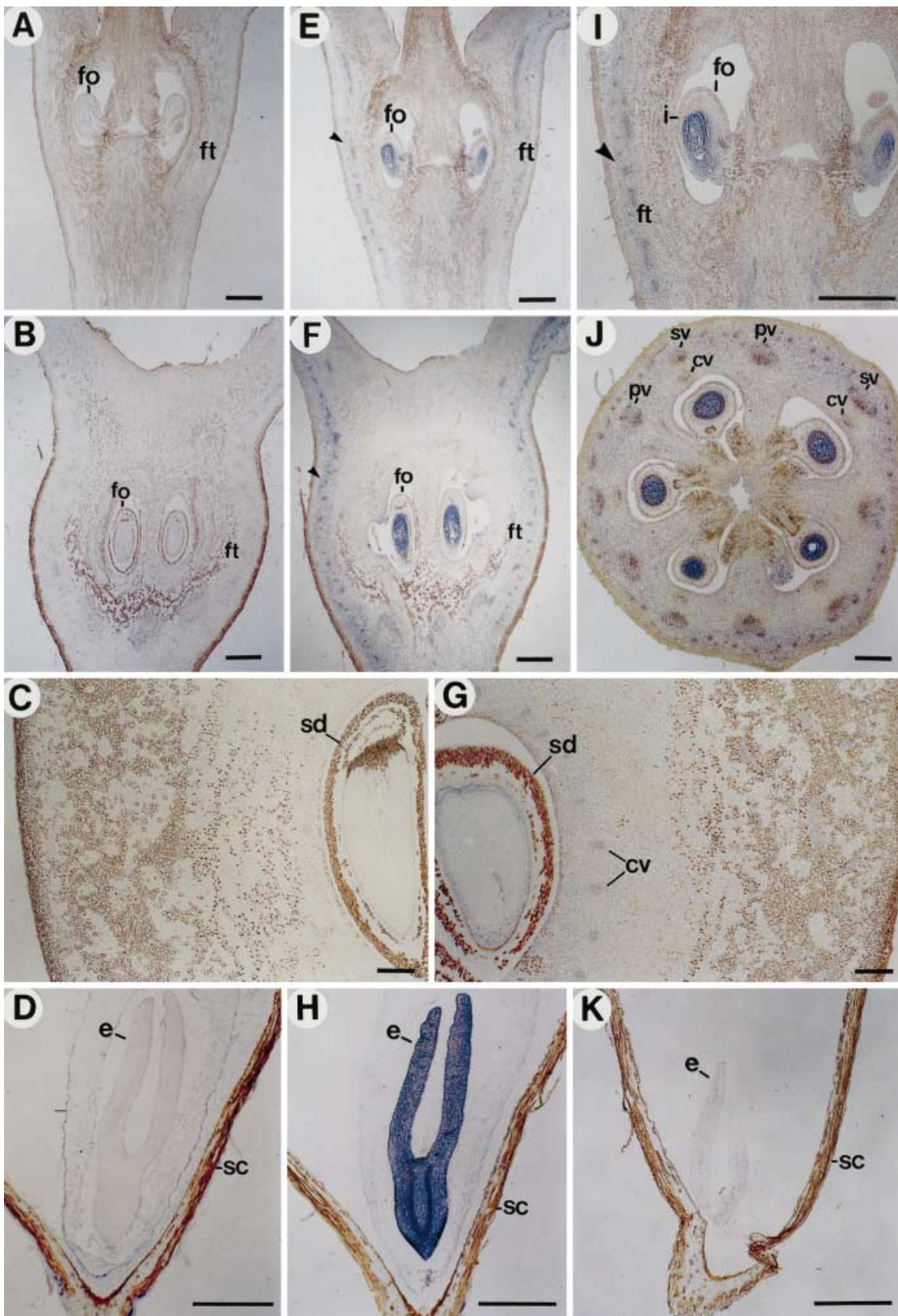
*MdMADS3**MdMADS4*

Fig. 6A–K. Patterns of *MdMADS3* and *MdMADS4* RNA expression in apple fruits during fruit set and growth. Sections of fruit were hybridized with antisense-RNA of *MdMADS3* (A–D), antisense-RNA of *MdMADS4* (E–J), and sense-RNA of *MdMADS4* (K). The transcript signal is blue. Bars = 500 μ m. **A,E,I** Longitudinal sections of one-week-old fruit after anthesis. **B,F,J** Longitudinal sections (**B,F**) and cross-section (**J**) of two-week-old fruit after anthesis. **C,G** Longitudinal sections of four-week-old fruit after anthesis. **D,H,K** Longitudinal sections of the seed in six-week-old fruit after anthesis. The *arrowheads* in **E,I**, and **F** indicate the vascular bundles assigned to the floral tube. *cv*, carpellary vascular bundles, *e*, embryo; *fo*, fertilized ovule; *ft*, floral tube; *i*, integument; *pv*, petal vascular bundle; *sc*, seed coat; *sd*, seed; *sv*, sepal vascular bundle

MdMADS4 probes as molecular markers. At the time of fruit set, *MdMADS4* was highly expressed in the vascular bundles assigned to the floral tube, which later form fruit flesh. Also, *MdMADS4* was expressed in the carpellary vascular bundles, which later form the core of the fruit. Considering that *MdMADS4* acts as a transcription factor, and that active expression of *MdMADS4* occurs in the vascular bundles assigned to the floral tube and the carpellary vascular bundles, *MdMADS4* may be an important factor contributing to the make-up of the fleshy tissues and core of young fruit. Active cell division in the flesh is usually limited to an initial period of a few weeks after anthesis (Coomb 1976). We have seen a dramatic decrease in *MdMADS4* expression in the fruit at four weeks after anthesis.

Unlike the pome fruits of the apple, the fruit of *Arabidopsis* develops from a gynoecium consisting of two carpels, and is known as a silique. A mutant strain of the *AGL8/FRUITFULL* MADS-box gene displays a lack of coordinated growth of the fruit tissues that leads to crowded seeds, suggesting that *AGL8/FRUITFULL* regulates the transcription of genes required for cellular differentiation during fruit development (Gu et al. 1998). Double recessive mutants of *AP2* and *AG* show a homeotic conversion with the reduction of carpels into leaf like structures (Yanofsky et al. 1990; Bradley et al. 1993), suggesting their role in fruit development (Gillaspay et al. 1993). Analysis of the *AGL2* expression patterns in wild-type flowers and the three floral homeotic mutants suggests that *AGL2* may play a fundamental role in establishing all floral organs, ovules, seed coats, and developing embryos (Flanagan and Ma 1994).

The *MdMADS4* gene was expressed at a high level in the developing ovules around the time of full bloom. After fertilization, *MdMADS4* continued to be expressed in the nucellus and the embryo sac of the fertilized ovule. It has been generally accepted that the presence of fertilized ovules triggers the fruit development (fruit set) (Costa Tura and Mackenzie 1990). The fact that *MdMADS4* is expressed in the embryo is not so surprising, since cell proliferation and differentiation in the fruit tissue are temporally coordinated with the mitotic activity in the developing seed and in the developing embryo (Coombe 1976).

Apples are perennial in temperate climates. Flower bud initiation and differentiation in apple trees occur in

the previous growing season, and the fruit set and growth occur in the following year. It has been generally accepted that the development of flower buds is highly correlated with the future fruit set and growth (Fulford 1966; Buban and Faust 1982; Lauri et al. 1996). The development of flower buds is thus used to estimate future yields in fruit-growing practice (Fulford 1966; Buban and Faust 1982; Lauri et al. 1996). However, little is known about the genetic and molecular control of the coordinated flower bud development and the fruit set. In the present study, we have shown that the *MdMADS4* gene, which shares high sequence similarities with members of the *AGL2* subfamily and shows expression patterns related to those of *AGL2* of *Arabidopsis*, plays a role in fruit development as well as in flower development. The spatial and temporal expression patterns of *MdMADS4* can provide good accounts at the genetic and molecular levels of the important events taking place in the flower and of the developmental events leading to the setting of fruit.

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