Seven MADS-box Genes in Apple are Expressed in Different Parts of the Fruit

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ABSTRACT. To study the role of MADS-box genes in developing apples (*Malus ×domestica* Borkh.), clones corresponding to seven different genes, *MdMADS5* to *MdMADS11*, were isolated from a 2-day-old apple cDNA library. Through DNA sequence comparison, six genes were classified into the *APETALA1* (*API*) group and one gene, *MdMADS10*, into the *AGAMOUS* (*AG*) group. Six of the genes, *MdMADS5* to *MdMADS10*, were found to be preferentially expressed in fruit following pollination. These genes also showed differential expression patterns in core, cortex and skin of young fruit. For instance, *MdMADS5*, which is highly homologous to *API*, showed preferential expression in the cortex and skin tissues while *MdMADS10*, which is highly homologous to *AGL11*, showed exclusive expression in the core tissues. The gene *MdMADS11* showed a similar expression level and pattern in flowers, fruit at several early developmental stages, and for different fruit tissues. The range of expression patterns suggests that the genes play different roles in apple development.

MADS-box genes have been identified from a wide range of eukaryotic organisms including yeast, insects, mammals and plants (Shore and Sharrocks, 1995). They form a superfamily whose members share a highly conserved domain of 56 amino acids, named MADS-box after four of the originally cloned members: MCM1, AG, DEFA and SRF (Shore and Sharrocks, 1995). The genes in this family encode transcription factors that have diverse roles in different developmental processes of eukaryotic organisms (Shore and Sharrocks, 1995; Yanofsky et al., 1990).

In plants, MADS-box genes have been shown to play fundamental roles in flower development by controlling floral meristem and floral organ identity (Coen and Meyerowitz, 1991; Davies and Schwarz-Sommer, 1994; Weigel and Meyerowitz, 1994). *APETALA1* (*API*), an arabidopsis [*Arabidopsis thaliana* (L.) Heynh] meristem identity gene, controls the transition from vegetative meristem to floral meristem (Mandel et al., 1992; Mandel and Yanofsky, 1995). Homologs to *API* also have been cloned from *Antirrhinum majus* L. (Huijser et al., 1992) and maize (*Zea mays* L.) (Mena et al., 1995). The floral organ identity MADS-box genes have been grouped into three different classes by function according to the proposed ABC model (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). In summary for arabidopsis, the class A gene *API* specifies sepal formation; classes A and B [*APETALA3* (*AP3*) and *PISTILLATA* (*PI*)] genes together specify petal formation; classes B and C [*AGAMOUS* (*AG*)] genes together are required for stamen formation; and class C genes alone control carpel formation (Weigel and Meyerowitz, 1994). More recently, a class D gene (*FBP11*) has been described in petunia (*Petunia hybrida* Vilm.) controlling ovule development (Angenent et al., 1995; Colombo et al., 1995).

The relatively high DNA sequence conservation within the MADS-box of these genes has permitted isolation of additional gene members from arabidopsis by means of cross hybridization using homologous DNA probes or through PCR amplification using degenerative primers (Ma et al., 1991; Rounsley et al., 1995). Similar methods have been used to clone MADS-box genes from

a variety of different plant species including tomato (*Lycopersicon esculentum* Mill.) (Pnueli et al., 1991), rice (*Oryza sativa* L.) (Chung et al., 1994; Kang et al., 1995), Norway spruce [*Picea abies* (L.) Karsten] (Tandre et al., 1995), *Sinapis alba* L. (Menzel et al., 1996), potato (*Solanum tuberosum* L.) (Kang and Hannapel, 1996), *Pinus radiata* D. Don (Mouradov et al., 1996), *Sorghum bicolor* (L.) Moench (Greco et al., 1997), apple (Sung and An, 1997), and cucumber (*Cucumis sativus* L.) (Filipecki et al., 1997). Although the majority of MADS-box genes isolated so far show floral specific expression, additional genes have alternative expression patterns in a range of plant tissues including: leaves, roots, embryos (Rounsley et al., 1995), fruit (Sung and An, 1997), embryogenic callus (Filipecki et al., 1997) and potato tubers (Kang and Hannapel, 1996). This diverse range of expression patterns for MADS-box genes suggest that they are likely to play roles in many other aspects of plant development in addition to their role in flowering.

Following floral organ specification and anthesis, fruit development starts with a rapid cell division phase which is normally stimulated by pollination and fertilization, and continues through a cell expansion phase to ripening (Gillaspy et al., 1993). Relatively few studies have been undertaken to characterize the molecular basis of fruit development. Most molecular studies on fruit have concentrated on fruit ripening through the characterization of ethylene biosynthesis genes (Bolitho et al., 1997; Grierson and Fray, 1994; Hamilton et al., 1990) and cell wall softening genes such as polygalacturonase (Kramer and Redenbaugh, 1994; Watson et al., 1994). A few cDNA clones have been isolated from young fruit cDNA libraries using differential screening (Dong et al., 1997; Ledger and Gardner, 1994; Santino et al., 1997) or differential display techniques (Tieman and Handa, 1996). However, the role of most of these genes in fruit development remains to be determined. Processes occurring during the cell division phase are critical to determine final fruit size, shape and quality. We were interested in isolating genes contributing important roles in the cell division phase of apple development and have chosen to work on MADS-box genes because they influence biological development through control of cell fate and differentiation (Shore and Sharrocks, 1995).

Apple falls into the pome fruit category. The fruit core develops from an inferior ovary which is surrounded by the floral tube developing into fruit flesh (Pratt, 1988). This type of fruit structure

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differs from tomato, a model system often used in studies of fruit development, where the fruit develops from ovary tissue only (Gillaspay et al., 1993). Developmental processes in apples, particularly in the cortex tissue, are likely to be very different from those in tomato. Therefore, molecular studies in apple development may be useful in further defining these differences. In this report, we describe the isolation of seven MADS-box genes from a cDNA library of young apples and provide evidence for their involvement in fruit development.

Materials and Methods

PCR AMPLIFICATION OF MADS-BOX DNA FRAGMENTS. Total RNA was isolated from 'Granny Smith' apples at 2 d after pollination using the method described by Chang et al. (1993). Poly(A) mRNA was purified from the total RNA using the mRNA Purification Kit (Pharmacia, Sweden). cDNA was synthesized from the mRNA using the ZAP cDNA Synthesis Kit (Stratagene). MADS-box DNA fragments were amplified from templates of cDNA using two degenerative PCR primers: 5'-CGGAATTCATGGGNGMGGNAARRT-3', 5'-CGGGATCCACATCNGCR-TCRCANA-3' (N = ATGC, M = AC, R = AG, Y = CT). The primers were designed according the conserved amino acid sequences MGRGKV/I and LCDAEV in the MADS-box domain. The underlined *Eco* RI and *Bam* HI sites were used for cloning the PCR products. The PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min; then 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min; and with a final extension of 5 min at 72 °C. Several bands were detected from the PCR on agarose gels and DNA in a band of the expected size (145 bp) was cloned into Bluescript SK (Stratagene) following *Eco* RI and *Bam* HI digestion.

CONSTRUCTION OF A cDNA LIBRARY AND SCREENING FOR MADS-BOX CLONES. A ZAPII (Stratagene) library was constructed using cDNA as used for the PCR amplification. About 1×10^5 phage colonies from the library were then screened on Hybond-N⁺ nylon membranes (Amersham, England) using a mix of seven different ³²P-dCTP labelled MADS-box DNA fragments. The membranes were hybridized in 0.5 M NaPO₄ buffer (pH 7.2) with 1 mM EDTA and 7% SDS at 65 °C and washed using 0.4× SSC and 0.2% SDS at 65 °C.

DNA SEQUENCE DETERMINATION. Nucleotide sequences of MADS-box clones were determined using the automatic sequencer ABI PRISM model 377 with universal forward and reverse primers. To

obtain complete sequences, cDNA clones were further subcloned in Bluescript and both DNA strands were sequenced.

COMPARISON ANALYSES OF DNA SEQUENCES. Nucleotide and predicted amino acid sequences for one *Pinus radiata* and nine different arabidopsis MADS-box genes were selected from GenBank. These sequences were then used together with the seven apple genes, MdMADS5 to MdMADS11, to produce both amino acid and nucleotide sequence alignments using the PILEUP program of the GCG package (version 9; Genetics Computer Group, Madison, Wis.). The nucleotide sequence alignment was visually refined using the amino acid alignment as a guide. The first and second codon positions in the MADS-box and K-box were used for phylogenetic analysis using parsimony methods in the PAUP program (Swofford, 1993). The tree was constructed from the results of 100 bootstrap replicates using the PAUP heuristic search.

NORTHERN ANALYSIS USING MADS-BOX CLONES ON APPLE TISSUES. Total RNA was isolated as in Chang et al. (1993) from 'Granny Smith' apple tissues. Northern blots were prepared by transferring RNA to Hybond-N⁺ nylon membranes following agarose gel electrophoresis under denaturing conditions (Dong et al., 1997). The first northern blot contained RNA isolated from expanding leaves, unopened flowers, and fruit at 2 d and 1, 4, and 8 weeks following hand-pollination. At 4 weeks after pollination, apples are large enough to allow for easy separation into the three main tissue types, namely; core, cortex, and skin. In order to determine the relative gene expression patterns for apple MADS-box genes within fruit, the second northern blot was made using RNA isolated from these three fruit parts. Northern blots were sequentially probed with ³²P-dCTP labelled MADS-box cDNA clones lacking the MADS-box sequence to significantly reduce cross hybridization. Finally, members were hybridized to an 18S rDNA probe from apple (Simon and Weeden, 1992) for assessment of even loadings of RNA. The hybridization and washing conditions were the same as those in cDNA library screening. Membranes for reprobing with different MADS-box gene clones and rDNA were stripped by pouring on boiling 0.5% SDS and allowing it to cool to room temperature, as recommended by the manufacturer (Amersham, England).

Results

ISOLATION AND SEQUENCE ANALYSIS OF SEVEN DIFFERENT APPLE MADS-BOX GENES. DNA fragments of 145 bp were amplified from

Table 1. Size of cloned apple MADS-box genes and comparison of their deduced amino acid sequence to putative homologues.

Clone	EMBL accession no.	No. bp	No. AAs	Sequence identity with related genes (%) ²			
				Gene	MADS	K-box	Overall
MdMADS5	AJ000759	1055	239	AP1	94.6	71.6	71.0
MdMADS6	AJ000760	972	248	PrMADS1	94.6	66.5	50.7
				MdMADS7	91.1	80.6	83.1
MdMADS7	AJ000761	1177	248	PrMADS1	96.4	77.6	71.8
MdMADS8	AJ001681	1291	246	MdMADS1	100	100	99.6
				AGL2	100	91.0	80.7
				AGL4	96.4	88.1	74.3
				MdMADS9	98.2	94.0	93.4
MdMADS9	AJ001682	964	246	AGL2	98.2	86.6	76.3
				AGL4	98.2	82.1	73.8
MdMADS10	AJ000762	877	207	AGL11	96.4	62.7	78.2
				FBP7	91.1	53.7	65.9
MdMADS11	AJ000763	1240	243	AGL6	96.4	76.0	67.0

²The numbers are percentages of amino acid identities in the MADS-box domain, in the K-box domain and over the full gene.

apple cDNA using degenerative PCR primers against conserved sequences in the MADS-box. After these DNA fragments were cloned, nine random clones were sequenced. Seven clones were

Fig. 1. Sequence alignments between predicted MADS-box proteins. (a) Alignment between the deduced peptides MdMADS5 and AP1 (Mandel et al., 1992) of Arabidopsis. (b) Alignment between the deduced peptides MdMADS8, MdMADS9 and AGL2 (Ma et al., 1991) of Arabidopsis. (c) Alignment between the deduced peptides MdMADS10 and AGL11 (Rounsley et al., 1995) of Arabidopsis. The N-terminal MADS-box and the central K-box are underlined. Asterisks indicate amino acid identity, while dots indicate gaps introduced to maximise alignments.

found to contain independent MADS-box DNA sequences, while one showed strong homology to a histone H2A gene, and the remaining clone had no homology to any entry within the database. Using a mixture of the seven different MADS-box cloned fragments to screen a cDNA library prepared from apple mRNA at 2 d after pollination, 11 positive cDNA clones were isolated, representing seven independent genes as determined by partial sequencing. Only one of the cDNA clones had an identical sequence to one of the seven PCR clones.

The entire nucleotide sequences of the seven cDNA clones containing different MADS-box sequences were determined and submitted to the EMBL database. These clones were named as *MdMADS5* to *MdMADS11* (*Malus domestica* MADS) following the numbering system used in a recent study of apple MADS-box genes (Sung and An, 1997). All seven clones contain a putative open reading frame, a 3' untranslated region, and a poly(A) tail. There was a 5' untranslated region between 11 bp and 374 bp for *MdMADS5*, *MdMADS7*, *MdMADS8*, *MdMADS10*, and *MdMADS11*. The size of each clone and their deduced peptides are summarized in Table 1. All deduced peptides contain the MADS-box domain (although *MdMADS6* and *MdMADS9* lack three and four amino acids, respectively, at the N-terminus being only partial sequences), the K-box domain, an intervening region between the MADS-box and the K-box, and a C-terminal region. These features confirm that the clones belong to the MADS-box gene family.

Comparisons of deduced amino acid sequence revealed a high level of sequence similarity between *MdMADS6* and *MdMADS7*, as well as between *MdMADS8* and *MdMADS9* (Table 1, Fig. 1b). Comparisons made with the predicted amino acid sequence from each of the seven apple MADS-box genes and the GenBank database using BLAST searching revealed that *MdMADS5* had highest homology with AP1 (Mandel et al., 1992); *MdMADS6* and *MdMADS7* with PrMADS1 (Mouradov et al., 1996); *MdMADS8* and *MdMADS9* with AGL2 and AGL4 (Ma et al., 1991); *MdMADS10* with AGL11 (Rounsley et al., 1995) and FBP7 (Angenent et al., 1995); and *MdMADS11* with AGL6 (Ma et al., 1991). The percentage of amino acid sequence identity between these genes over the entire sequence, in the MADS-box domain and in the K-box domain, are listed in Table 1.

An amino acid alignment of *MdMADS5* and AP1 showed that

Fig. 2. Phylogenetic tree of selected plant MADS-box genes using parsimony analysis. The numbers next to the nodes give bootstrap values from 100 replicates. Nodes with <50% bootstrap support are collapsed.

a 17 amino acid segment in the C-terminal region of AP1 was apparently absent in MdMADS5 although these two proteins are highly conserved in the MADS-box and in the C-terminus (Fig. 1a). MdMADS8 and AGL2 showed an overall amino acid identity of 80.7%, with total sequence identity in MADS-box domains and over six amino acids in the C-terminus (Table 1, Fig. 1b). MdMADS10 contained a deletion of 17 amino acids, mostly in the K-domain, when compared to AGL11 (Fig. 1c).

Most plant MADS-box genes have been classified into three groups following phylogenetic analysis: the *AP1* group, *AG* group and *PI/AP3* group. In general, genes within each group have similar function and expression patterns (Purugganan et al., 1995; Rounsley et al., 1995). Using a similar analysis procedure, we classified *MdMADS10* into the *AG* group and the six remaining apple genes, *MdMADS5* to *MdMADS9* and *MdMADS11* into the *AP1* group (Fig. 2). Using our screening procedure, we obtained no apple genes from the *PI/AP3* group.

TIMING OF APPLE MADS-BOX GENE EXPRESSION DURING FRUIT DEVELOPMENT. Expression was not readily detected in apple leaf tissue for any of these genes. The apparent mRNA level for *MdMADS5* to *MdMADS10* was lower in unpollinated flowers but markedly increased in fruit tissue 2 d after pollination (Fig. 3).

Fig. 3. Expression of seven apple MADS-box genes as determined by northern analysis. Total RNA was used from young leaves (L), flower buds (F) and fruit at 2 d (2d), and 1, 4 and 8 weeks (1w, 4w, 8w) after pollination. One northern blot was sequentially probed with gene-specific probes for the genes shown at left. The rDNA probe was used as a loading control.

Expression of *MdMADS5* was further stimulated at 1 week after pollination, possibly a result of fertilization since this process in apple takes approximately one week to occur (Williams, 1965). *MdMADS5* remained at the same expression level at 4 and 8 weeks. *MdMADS6* showed a relatively constant expression level between 2 d and 8 weeks, while the expression of *MdMADS7* was strongest at 4 and 8 weeks compared to 2-d and 1-week fruit. *MdMADS8*, *MdMADS9*, *MdMADS10* and *MdMADS11* shared a similar expression pattern with the highest transcript level being detected at 2 d after pollination followed by a gradual decrease in expression. Hybridization result to an 18S rDNA probe showed even loadings of RNA samples on the northern blot.

APPLE MADS-BOX GENES ARE EXPRESSED IN DIFFERENT PARTS OF THE FRUIT. The seven MADS-box genes were differentially expressed in fruit tissue, producing five different expression patterns (Fig. 4). *MdMADS5* was strongly expressed in cortex and skin but very weakly expressed in the core. *MdMADS6* and *MdMADS7* had a higher expression level in the cortex and skin than in core although significant levels of expression were found in the core tissue. In contrast, the expression of *MdMADS8* and *MdMADS9* was stronger in the core and cortex but weaker in skin. *MdMADS10* expression could only be detected in the core while *MdMADS11* was evenly expressed in the three different fruit tissues (Fig. 4).

Fig. 4. Expression of seven apple MADS-box genes in different parts of the apple as determined by northern analysis. Total RNA was used from core (Cr), cortex (Cx) and skin (Sk) of apples at 4 weeks after pollination. One northern blot was sequentially probed with gene-specific probes for the genes shown at left. The rDNA probe was used as a loading control.

Discussion

Apples are pome, having a fleshy cortex in addition to a core derived from the ovary. The cortex tissue develops from the floral tube, which consists of the fused bases of the sepals, petals and stamens according to the appendicular hypothesis [for review, (Pratt, 1988)]. In *Arabidopsis* *AP1* is expressed in young flower primordia to specify the identity of flower meristems and later in sepals and petals to determine the development of these two floral organs (Mandel et al., 1992). The expression of the *AP1*-like gene *MdMADS5* in apple cortex and skin is consistent with the probable origin of these tissues from sepal and petal bases. Of the seven MADS-box clones isolated, six were classified into the *AP1* group and one into the *AG* group (Fig. 1). From the additional six MADS-box gene fragments amplified from apple cDNA using degenerative PCR primers, five fit into the *AP1* group and one into the *AG* group (data not shown). However, we have not been able to isolate MADS-box genes with significant sequence identity to *AP3* or *PI*, which are both expressed in the petals and stamens (Goto et al., 1993; Jack et al., 1992). If petals, stamens and sepals contribute equally to apple cortex formation, we would have expected to detect some members of the *AP3/PI* group as the degenerative primers used should be able to amplify *AP3*-like or *PI*-like genes. These data suggest that the cortex tissue develops mainly from the bases of sepals and that the cells of petals and stamens have little or no contribution to cortex formation.

The putative products of *MdMADS8* and *MdMADS9* show extensive similarity to *AGL2* of *Arabidopsis* (Ma et al., 1991). *In situ* hybridization experiments have previously shown that the *AGL2* transcript is present in all four whorls of the flower during floral organ meristem development and during morphological differentiation of organs, but absent at the final maturation phase of development. The *AGL2* gene also is expressed early in seed and embryo development. Flanagan and Ma (1994) proposed that *AGL2* functions in the early development of all floral organs and ovules. Within the coding region, *MdMADS8* was almost completely identical to the previously isolated apple MADS-box gene *MdMADS1* (Sung and An, 1997), with only one nucleotide differing. The nucleotide sequence of *MdMADS8* was 346 bp longer in the 5' untranslated region and 46 bp shorter in the 3' untranslated region compared to *MdMADS1*, in addition to one nucleotide difference in the 3' untranslated region. *MdMADS1* and *MdMADS8* are likely to represent the same gene with the differences likely due to different apple cultivars [their Fuji (Sung and An, 1997) vs our Granny Smith] or to variations in cDNA synthesis and sequence analysis. Sung and An (1997) have shown that *MdMADS1* has a similar expression to *AGL2* in floral organs. Our data show that the expression level of *MdMADS8* and *MdMADS9* is high in fruit tissue after pollination and then gradually decreases (Fig. 3). It is, therefore, possible that *MdMADS8* and *MdMADS9* play an important role in the early process of apple development.

The predicted protein sequence of *MdMADS10* has a high level of homology to *FBP7* and *AGL11*, which have a role in the control of ovule and seed development (Angenent et al., 1995; Colombo et al., 1995; Colombo et al., 1997; Rounsley et al., 1995). *MdMADS10* mRNA was only detected in the fruit core (Fig. 4), which develops from ovary tissue and contains the seeds. No signal was detected in the cortex sample that included a small amount of outer core tissue. This suggests that *MdMADS10* is expressed in the central core tissue, possibly in the seed. Expression of *MdMADS10* mRNA in apple ovule integuments and seed coat was detected by *in situ* hybridization techniques (Yao, unpublished data). *MdMADS10* may, therefore, play an important role in ovule

and seed development. Since seed development has a significant impact on fruit development (Gillaspy et al., 1993), manipulation of *MdMADS10* expression may also influence this process.

Previous studies on plant MADS-box genes have concentrated largely on flower development (Davies and Schwarz, 1994; Theißen and Saedler, 1995). In one such study, an apple MADS-box gene isolated from a flower bud cDNA library was found to be expressed in fruit tissue (Sung and An, 1997). In our work, we are interested in characterizing the role of MADS-box genes in apple development and have begun by isolating seven different MADS-box clones from a cDNA library constructed from 'Granny Smith' apples at 2 d after pollination. Six of these genes were preferentially expressed in fruit tissue 2 d following pollination and they were found to be expressed in different parts of the fruit. On the basis of temporal and spatial differences in their expression, we postulate that these genes play several different roles in apple development.

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