Molecular Cloning and Characterization of a MADS-Box cDNA Clone of the Fuji Apple

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A cDNA clone, MdMADS1, containing MADS domain was isolated from the Fuji apple. The gene was expressed in all floral organs and young fruits but not in leaves. The expression was higher at the early stages of flower and fruit developments, suggesting that MdMADS1 plays a major role in the initiation of reproductive organ developments.

Key words: Floral bud — Fruit — MADS-box gene — Malus × domestica — RNA hybridization — RT-PCR.

The study of flower development has rapidly progressed as genetic analysis has elucidated a number of homeotic genes which are required for normal flower development (Coen and Meyerowitz 1991, Ma 1994). The isolation of the first plant homeotic genes, DEFICIENS (DEF) of Antirrhinum majus (Sommer et al. 1990) and AGAMOUS (AG) of Arabidopsis thaliana (Yanofsky et al. 1990), led to the subsequent isolation of a number of MADS-box genes from a variety of plant species (Davies and Schwarz-Sommer 1994). The majority of the plant MADS-box genes function as floral meristem or floral organ identity genes. Meristem identity genes, such as SQUAMOSA (SQUA) from Antirrhinum and APETALA1 (API) from Arabidopsis, are involved in establishing the identity of the floral primordium (Huijser et al. 1992, Mandel et al. 1992). Floral organ identity genes, such as DEF and AG, interact in a combinatorial manner during development to manifest the identity of flower organs (Weigel and Meyerowitz 1994).

During the past year, the function of the MADS-box gene has gone far beyond that of floral meristem-identity and floral organ-identity genes in the classic models of Arabidopsis and Antirrhinum. Similar genes have now also been studied in other dicotyledonous plants (Angenent et al. 1993, Hardenack et al. 1994), and very similar structures and expression patterns have been found in more distantly related species, such as monocots (Chung et al. 1994, 1995, Fischer et al. 1995, Kang et al. 1995) and gymnosperms (Strauss et al. 1995, Tandre et al. 1995). The MADS-box multigene family can be divided into several subfamilies according to the primary sequences and expression patterns. Because of a general co-evolution between the MADS-box and the rest of the gene, the MADS-box sequence alone quite reliably indicates the membership of a gene to a certain subfamily. This information has lead the MADS-box gene to be considered as a model for the study of evolution (Theissen and Saedler 1993).

In contrast to the wealthy amount of information about the regulatory genes controlling floral meristem and floral organ development in annual plants, very limited data is available concerning these genes in woody plants. To our knowledge, there has been no report on the molecular study of the floral development in the fruit trees of the Rosaceae family. Furthermore, the homeotic genes of floral development in the apple have not yet been investigated. In this study we report the isolation of an apple MADS-box homologue and the examination of its expression pattern during floral and fruit development in the Fuji apple.

A AZAP II cDNA library that was prepared from immature floral bud mRNAs was screened with a rice MADS-box clone, OsMADS1 (Chung et al. 1994). We have shown earlier that ectopic expression of the gene resulted in early flowering in transgenic tobacco. The OsMADS1 transcript becomes detectable in the early floral primordia and the expression continues throughout the entire flower development. In order to reduce false positives, a 430-bp Naelf-BglII fragment carrying the conserved MADS-box region was used as a probe. By screening 200,000 plaques of the cDNA library, about 60 positive clones were detected at the first round of hybridization. This indicated that MADS box genes are highly expressed during apple flower development. Thirty plaques that showed a positive signal at the second round of hybridization were selected for further characterization. Restriction enzyme mapping of the inserts and partial sequencing of the ends revealed that these clones could be grouped into four independent cDNAs that have the conserved MADS-box domain. They were designated MdMADS1-MdMADS4. In this study, we report the characteristics of MdMADS1. The study on the rest of the MdMADS genes are in progress and will be published else-
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where when the work is completed.

DNA sequence analysis showed that the cDNA of MdMADS1 contains an open reading frame of 246 amino acid residues with a 29-bp 5' untranslated region and a 222-bp 3' non-coding region (Fig. 1). The deduced amino acid sequence contains the conserved MADS-box domain between the amino acid residues 2 and 57 (Fig. 1). The K-box, another conserved region among MADS-box proteins, is located between the residues 93 and 158 (Fig. 1).

Sequence comparison of MdMADS1 with other MADS-box proteins shows that MdMADS1 is 79% identical to the AGL2 and 74% to the AGL4 of A. thaliana. In the MADS-box and K-box regions, the amino acid sequence identity between MdMADS1 and AGL2 is 100% and 92.4%, respectively (Fig. 2). In addition, MdMADS1 shows an extensive similarity to the DEFH49 (70%) and FBP2 (65%). These proteins belong to the AGL2 subfamily of both monocot and dicot plants. Within the

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**Fig. 1** Nucleotides and deduced amino acid sequences of MdMADS1. The MADS-box and K-box regions are underlined. The restriction enzyme sites PstI, EcoO109I used for the generation of the probe have been double underlines. The termination codon is marked with an asterisk. The primers sequences used for the RT-PCR experiment are represent as dot-lines above the sequences. The positions of nucleotides and amino acids are shown on the left and right, respectively. The sequence data will appear in the DDBJ/EMBL/GenBank Databases under the accession number U78947.
MADS-box region, there is at most two amino acid replacements. These levels of homology were higher than those between AG and PLE which are functionally identical MADS-box proteins of A. thaliana and A. majus. Such a high degree of homology indicates that MADS-box genes in Rosaceae have co-evolved with other higher plant species.

It is well established that a large number of MADS-box genes are present in each plant species. Therefore, it is necessary to use a gene-specific probe in order to study the expression pattern of a particular MADS-box gene. Sequence comparison of MADS-box genes showed that there is a significant homology in the MADS-box and K-box regions and that the 3' portion of the genes is the least con-

Fig. 2 Comparison of MdMADS1 protein with other MADS-box proteins in the AGL2 subfamily. The alignment was done using the FastDB program (IntelliGenetics, U.S.A.). A. Sequence alignment of the MADS-domain. Shown here are the MADS-box sequences for: Malus domestica MdMADS1 (Fig. 1), Arabidopsis proteins AGL2 (accession number M55551), AGL3 (S79244) and AGL4 (M55552), Antirrhinum protein DEFH49 (X95467), petunia protein FBP2 (M91666), Pinus radiata proteins PrMADS1 (U42399) and PrMADS2 (U42400) and the rice protein OsMADS1 (L34271). The asterisks represent amino acid residues identical to the corresponding ones in MdMADS1. The numbers at the left represent the positions of the first amino acid residue shown for each sequence. B. Alignment of the K-domains. The asterisks and the numbers at the left are as represented in A. C. C-terminal of MADS-box proteins. The last ten amino acid residues are shown. The left numbers are the positions of the first amino acid residues shown for each sequence. The numbers at the right represent the percentage of the total number of identical amino acid residues with MdMADS1.
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Fig. 3 DNA blot analysis of apple genomic DNA probed with MdMADS1. Genomic DNA was digested with EcoRI (E), HindIII (H), or BamHI (B), and hybridized with a probe prepared from a 223-bp PstI-EcoO1091 fragment containing the 3' region of the clone. The positions of PstI digested λDNA size markers are also indicated.

Fig. 4 Analysis of MdMADS1 transcript during flower development. A. The RNA blot assay. The upper band is indicated by arrowhead. Lane 1, young leaf; lane 2, stage 1 (bud length = 4 to 5 mm; lane 3, stage 2 (bud length = 7 to 8 mm); lane 4, stage 3 (petals begins to emerge from sepals); lane 5, stage 4 (mature flower); lane 6, stage 5 (post-anthesis flower); lane 7; sepals of mature flowers; lane 8, petals of mature flowers; lane 9, stamens of mature flowers; lane 10, carpels of mature flowers. B. Ethidium bromide staining of 28S and 18S rRNAs demonstrates equal amounts of RNA loading. Lane numbers mean the same as in A. C. The RT-PCR assay. The lane numbers mean the same as in A. M is λDNA digested with PstI. C is RT-PCR product (523-bp) of a control RNA.

served. The possibility of the 3' region of MdMADS1 as a gene-specific probe was examined by DNA blot analysis using the 223-bp DNA fragment between nucleotides 693 to 916. Genomic DNA blot analysis revealed that two EcoRI, two HindIII, and three BamHI fragments were hybridized with the probe (Fig. 3). These results indicate that there may be another gene that is highly homologous to MdMADS1 in the apple genome.

The floral development was divided into five stages. In stage 1, the floral bud emerged on the flank of the branch and the bud length was 4-5 mm. In stage 2, ten days after stage one, it became 7-8 mm long. Stage 3 was 20 days after stage 1 during which petals began to emerge from the sepals. Stage 4 was at the mature flower stage. At this stage, all of the floral organs had completed morphological differentiation. Stage 5 was at the post-anthesis of the flower. The anther wall was opened and stigma began to senescence.

RNA blot analysis was performed on the total RNA isolated from the Fuji apple using the 223-bp DNA fragment probe that was used for the DNA blot analysis (Fig. 4). The MdMADS1 RNA was not detectable in leaves (lane 1) while two distinctive transcripts were detected in all five stages of flower development (lanes 2-6) and all four organs (lanes 7-10). The level of upper band was strongest at stage 1 (lane 2) and receded as the flower developed, whereas the intensity of the lower band was the highest at stage 4 (lane 5). In a mature flower, the upper band was strongest in petals and stamens (lanes 8 and 9), whereas the lower bands was strongest in sepals (lane 7). Two distinctive bands were detected from most of the floral samples even though the probe was small and it was from the least conserved area of the MADS box gene. Since the apple is an inter-species hybrid, it is possible that the two transcripts were likely to be products of two closely related MADS-box genes which were originated from each parent. If this was the case, it is interesting to notice that the expression patterns of the two transcripts were different from each other. Considering that the two genes share a significant sequence homology, the difference in the expression patterns was unexpected. It is also possible that the two bands were due to alternatively spliced mRNAs from a single MADS-box gene. Such a case was reported from a maize MADS-box gene, ZEMa (Montag et al. 1995). The ZEMa transcripts are present in almost all maize tissues, but specific differentially spliced forms were accumulated preferentially in mature organs.
**Fig. 5** Analysis ofMdMADSl transcript during fruit development. A. The RNA blot assay. The upper band is indicated by arrowhead. Lane 1, stage 1 (post-anthesis flower); lane 2, style tissues of the post-anthesis flower; lane 3, receptacle tissues including ovary of the post-anthesis flower; lane 4, stage 2 (7-10 days after anthesis); lane 5, stage 3 (young fruits, 8-10 mm length). B. Ethidium bromide staining of 28S and 18S rRNAs demonstrates equal amounts of RNA loading. Lane numbers mean the same as in A. C. The RT-PCR assay. Lane numbers mean the same as in A. M is λDNA digested with *PstI*. C is RT-PCR product (523-bp) of a control RNA.

Since the expression patterns of the two transcripts were different from each other, it was necessary to identify which one was the MdMADSl transcript. Since the probe size was already small, it was difficult to design a smaller probe. Therefore, the RT-PCR approach was performed to increase the specificity. A set of primers was designed to amplify a 798-bp DNA fragment of theMdMADSl transcript. The sense primer sequence was 5'-TTTAGTAACTAAGGACATGG-3' (nucleotides 14 to 33; Fig. 1), and the antisense primer was 5'-TGAGTGATTAGAGGCACTG-3' (nucleotides 793 to 812; Fig. 1). The results from the RT-PCR analysis are shown in Figure 4C. This experiment revealed that the relative intensity of the 798-bp fragment in various samples was almost identical to that of the upper band in the Figure 4A, indicating that the band was a product of theMdMADSl gene. This analysis produced a single band of a fragment which was the expected length of approximately 800-bp, indicating that there was no alternative splicing within the coding region. However, it can not rule out the possibility of an alternative splicing involving the untranslated regions of theMdMADSl gene.

To understand the role of theMdMADSl gene in fruit development, RNA blot hybridization and RT-PCR were performed. The fruit development was divided into 3 stages. Stage 1 of the fruit development was at post-anthesis of the mature flower (the same as floral development stage 5). Stage 2 was 7 to 10 days after anthesis. The petals, stamens, and styles were detached from the fruit and the receptacles started swelling. Stage 3 was the young fruit stage. The fruits were 8-10 mm in length, and seeds were already differentiated.

Both analyses revealed that theMdMADSl transcript was present in both styles (Fig. 5, lane 2) and receptacles (lane 3) at stage 1. The transcript level was highest at the stage 2 (lane 4) and declined slightly at stage 3 (lane 5). These data suggest that MdMADSl is important for fruit development, especially during the initiation of fruit development.

TheMdMADSl gene expression pattern is similar to that ofAGL2 ofArabidopsis. During the early and intermediate stages of flower development, AGL2 is expressed at a high level in all four whorls of the flower. As the flower organs undergo the final elongation and maturation phase of development, AGL2 expression is dramatically reduced. Reduction of the AGL2 expression occurs first in the sepals and then in stamen and the petals of mature flowers. AGL2 expression is also high in developing ovules, embryos and seed coats (Flanagan and Ma 1994). However, the AGL2 transcript was detectable in leaves (Ma et al. 1991) whereas theMdMADSl transcript was not found in the vegetative organ.

Taken together, it appears that MdMADSl is a member of the AGL2 subfamily. Unfortunately, the function of the AGL2 subfamily is largely unknown. Transgenic phenotypes ofFBP2 andTM5 deficient plants suggest a role in mediating between meristem and organ identity genes (Angenent et al. 1994, Pnueli et al. 1994). We ectopically expressed theMdMADSl genes by placing the gene under the control of 35S promoter and transforming the chimeric gene into tobacco. However, we have not observed any alteration of flower or seed development (unpublished data). In order to reveal the functional role ofMdMADSl, it may be necessary to employ either antisense or co-suppression approaches in the homologous apple plant to achieve reduction of the gene expression.
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