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Characterization of *MdMADS2*, a Member of the *SQUAMOSA* Subfamily of Genes, in Apple[1]

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A MADS-box gene, *MdMADS2*, was isolated from the apple (*Malus* × *domestica* Borkh.) var Fuji and its developmental expression pattern was studied during flower development. *MdMADS2* shares a high degree of amino acid sequence identity with the *SQUAMOSA* subfamily of genes. RNA blot analysis showed that *MdMADS2* is transcribed through all stages of flower development, and its transcription was seen in the four floral organs. RNA in situ hybridization revealed that the *MdMADS2* mRNA is expressed both in the inflorescence meristem and in the floral meristem. The *MdMADS2* transcript was detected at all stages of flower development. Protein localization analysis showed that MdMADS2 protein was excluded from the stamen and carpel primordia, in which a considerable *MdMADS2* mRNA signal was detected. This indicates that posttranscriptional regulation may be involved in the *MdMADS2*-mediated control of flower development. Transgenic tobacco expressing the *MdMADS2* gene from the cauliflower mosaic virus 35S promoter showed early flowering and shorter bolts, but did not show any homeotic changes in the floral organs. These results suggest that *MdMADS2* plays an important role during early stages of flower development.

Flower formation in higher plants is a complex process controlled by genetic and environmental factors (Bernier, 1988; Yanofsky, 1995; Amasino, 1996; Levy and Dean, 1998). Much has been learned from genetic and molecular studies of floral meristem and floral organ formation in Arabidopsis and snapdragon. It was found that processes of flower development are controlled by MADS-box genes that encode proteins sharing similarity with transcription factors from yeast and mammals (Schwarz-Sommer et al., 1990). The plant MADS-box genes have a conserved DNA-binding domain called MADS (MCM1, AGAMOUS, DEFICIENS, and SRF) domain and a second conserved domain called K, which is involved in protein to protein interaction (Schwarz-Sommer et al., 1990; Ma et al., 1991; Davies et al., 1996).

The majority of plant MADS-box genes that have been characterized function as floral meristem or organ identity genes. The MADS-box genes, such as *AP1* (*APETALA1*) and *CAL* (*CAULIFLOWER*) from Arabidopsis (Irish and Sussex, 1990; Mandel et al., 1992; Weigel et al., 1992; Bowman et al., 1993; Kempin et al., 1995) and *SQUA* (*SQUAMOSA*) from snapdragon (Huijser et al., 1992), are characterized as floral meristem identity genes. The MADS-box genes, such as *AP3* (*APETALA3*), *PI* (*PISTILLATA*), and *AG* (*AGAMOUS*) from Arabidopsis (Yanofsky et al., 1990; Jack et al., 1992; Goto and Meyerowitz, 1994) and *DEF* (*DEFICIENS*), *GLO* (*GLOBOSA*), and *PLE* (*PLENA*) from snapdragon (Schwarz-Sommer et al., 1990; Sommer et al., 1990; Tröbner et al., 1992; Bradley et al., 1993), are characterized as floral organ identity genes. It became evident that several other MADS-box genes have more subtle functions that are associated with floral meristem and floral organ identity. These genes include *AGL2* (Flanagan and Ma, 1994), *AGL4* (Savidge et al., 1995) and *AGL9* (Mandel and Yanofsky, 1998) from Arabidopsis, *SaMADS D* from white mustard (*Sinapis alba*; Bonhomme, 1997), *TM5* from tomato (Pnueli et al., 1991, 1994), and *FBP2* from petunia (Angenent et al., 1992, 1994).

Recently, MADS-box genes have been isolated from woody plants, which include *DAL1* to *DAL3* from Norway spruce (Tandre et al., 1995, 1998), *EAP1* to *EAP3* and *egm1* to *egm3* from eucalyptus (*Eucalyptus glabra*) (Kyozuka et al., 1997; Southerton et al., 1998), *PrMADS1* to *PrMADS3* from Monterey pine (*Pinus radiata*) (Mouradov et al., 1998), and *MdMADS1* from Fuji apple (*Malus* × *domestica* Borkh. var Fuji; Sung and An, 1997).

Apple is one of the most economically important woody plant species, cultured for its valuable fruits. Fuji apple is the most important and widely cultivated commercial fruit in East Asia. The factors that affect the formation of flowers in apple trees are of particular interest in horticulture, but relatively little attention has been given to the molecular and genetic control of apple flower development. We previously isolated and characterized a MADS-box gene, *MdMADS1*, from Fuji apple, which was classified as a member of the *AGL2* subfamily (Sung and An, 1997). In the present study, we report characteristics of another MADS-box gene from Fuji apple, *MdMADS2*, which shows high sequence similarities to the *SQUA* subfamily genes.

MATERIALS AND METHODS

Plant Materials

The apple (*Malus* × *domestica* Borkh.) var Fuji was used in this study. Plant samples were provided by the Kyung-

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Abbreviations: DIG, digoxigenin; IPTG, isopropyl β-D-thiogalactopyranoside.

buk Provincial Rural Development Administration (Taegu, Korea).

Construction of cDNA Library and Isolation of *MdMADS2*

Young flower buds at 1 to 2 mm in length were used for construction of a unidirectional cDNA library according to the manufacturer's protocols (Stratagene). The initial plaque forming units were 1.9×10^6 and the average size of the inserts was 1.7 kb. The library was amplified once on agar plates and stored in a 7% DMSO solution at −70°C. *MdMADS2* cDNA clone was isolated according to the method of Sung and An (1997). Overlapping subclones were created in a pBluescript SK(−) vector (Stratagene), and nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a kit (Sequenase version 2.0, United States Biochemicals). Comparison of the deduced amino acid sequence was performed on GenBank databases and amino acid alignment was performed using the FastDB program (IntelliGenetics, Mountain View, CA).

RNA Isolation and RNA Blot Analysis

Total RNA was isolated from leaves, immature flower buds, mature flowers before anthesis, and mature, post-anthesis flowers of outdoor-grown trees. Mature flowers before anthesis were dissected into their individual floral organs (sepals, petals, stamens, and carpels), and total RNA was isolated from each. RNA was extracted with a solution containing 4 M guanidine isothiocyanate and further purified by ultracentrifugation in a 5.7 M CsCl solution (Sambrook et al., 1989). For RNA hybridization, 25 μg of total RNA from each sample was separated on an agarose-formamide gel. The gel was blotted onto a Hybond-N^+ nylon membrane (Amersham) and hybridized as described previously (Church and Gilbert, 1984) at 60°C for 16 h with the 548-bp DNA fragment between nucleotides 682 and 1,230 of the *MdMADS2* cDNA. The blot was washed twice with a solution containing 0.2× sodium chloride/sodium phosphate/EDTA buffer and 0.1% SDS for 10 min at room temperature, followed by two washes with the same solution at 55°C for 10 min each. The blot was exposed to x-ray film with an intensifying screen at −70°C for 3 d.

RNA in Situ Hybridization

The 321-bp 3′ region (nucleotides 682–1,005) of the *MdMADS2* cDNA was cloned into the pBluescript SK(−). This plasmid, pGA1526-3, was used as a template for synthesizing single-strand RNA. To generate an antisense-RNA probe, pGA1526-3 was linearized with *Bam*HI and an approximately 370-bp single-stranded RNA was synthesized by T7 RNA polymerase using a DIG RNA labeling kit (Boehringer Mannheim). To generate a sense strand probe, the pGA1526-3 plasmid was linearized with *Xho*I, and an approximately 350-bp DIG-labeled sense strand RNA was synthesized using T3 polymerase.

Flower buds at different developmental stages were fixed and paraffin embedded according to the method of Dixon et al. (1995). The samples were sectioned 10 μm thick, dewaxed, and rehydrated. The sections were incubated with proteinase K solution (100 mM Tris-HCl, pH 7.5, 50 mM EDTA, and 20 μg mL^{-1} proteinase K) at 37°C for 30 min and then acetylated with 0.25% acetic anhydride in 100 mM triethanolamine, pH 8.0, for 5 min at room temperature. The sections were washed with 2× SSC and dehydrated through an ethanol series. The sections were prehybridized in a hybridization solution (50% deionized formamide, 10% dextran sulfate, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10 mM $NaPO_4$, pH 8.0, 10 mM DTT, 5 mM EDTA, 1× Denhardt's solution, 500 μg mL^{-1} tRNA, 100 μg mL^{-1} salmon-sperm DNA, and 40 units mL^{-1} RNase inhibitor) at 45°C for 2 h, and hybridized with 0.8 μg mL^{-1} RNA probe at 48°C for 16 h. The sections were washed with 3× SSC for 5 min at room temperature and incubated with NTE buffer (500 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.5) containing 50 μg mL^{-1} RNase A at 37°C for 30 min. After RNase treatment, sections were washed with NTE buffer three times for 5 min at room temperature and followed by washing with 0.2× SSC at 60°C for 1 h.

For detection of hybridization signals, the sections were incubated in a color development solution (10% of polyvinyl alcohol [M_r 70,000–100,000], 100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mM 5-bromo-4-chloro-3-indolylphosphate, and 0.2 mM nitroblue tetrazolium) at 30°C for 8 to 12 h. The slides were extensively washed with distilled water and dehydrated and mounted with non-aqueous mounting medium using standard protocols. Slides were photographed on a microscope (Labophot-2, Nikon) using bright-field optics.

Antibody Preparation

To express a truncated form of the MdMADS2 protein (amino acid residues 66–256) lacking the MADS domain, the 802-bp *Bam*HI-*Xho*I fragment was cloned into the T7 expression vector pRSET C (Invitrogen, Carlsbad, CA). This construct was introduced into the *Escherichia coli* strain BL21 (pLysS) and protein was induced with 1 mM IPTG. The overexpressed MdMADS2 protein was purified on a Ni-affinity column (Invitrogen) according to the manufacturer's protocol. Isolated protein was further purified by electrophoresis on 12% SDS-PAGE as follows. After electrophoresis, the gel was soaked in cold 100 mM KCl and incubated at 4°C until protein bands were visualized. The protein band corresponding to the truncated MdMADS2 protein was excised from the gel and the protein was extracted with 100 mM KCl. The protein was concentrated with Centriprep columns (Amicon, Beverly, MA) with a cutoff size of 10 kD. Rats were immunized with 40 μg of protein per injection and were boosted a total of five times at 2-week intervals; the fifth boosting was done without adjuvant. Antibodies for the MdMADS3 (accession no. U78949), MdMADS4 (accession no. U78950), and OsMADS1 (accession no. L34271) were also prepared according to the same procedure described above. The antibodies were affinity purified using a western purification procedure (Burke et al., 1982).

Cross-reaction between the antibodies was examined by protein blot analysis. One microgram of purified MADS

(MdMADS2, MdMADS3, MdMADS4, and OsMADS1) proteins lacking the MADS domain were separated on a 12% SDS-PAGE and blotted onto a Hybond-C nitrocellulose membrane (Amersham). The membrane was incubated with MdMADS2 antibodies (1:5,000 dilution) and immunodetection was carried out using the secondary antibody (peroxidase-labeled affinity purified antibody to rat IgG [H+L]; KPL, Gaithersburg, MD) and a chemiluminescence system (Amersham). The membrane was then exposed to x-ray film for 10 s.

Protein Immunolocalization

For detection of immunoreactive proteins, the sections were treated with 3% H_2O_2 for 20 min at room temperature. The slides were washed for 10 min in PBS buffer (10 mM phosphate, pH 7.5, and 0.9% [w/v] NaCl) and incubated in a blocking solution (5% [v/v] normal rabbit serum and 0.5% [w/v] IgG-free BSA [A-9085, Sigma] in PBS buffer) for 2 h at room temperature. The sections were incubated with an avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA) to prevent nonspecific binding of avidin/biotin according to the manufacturer's protocols. The sections were briefly washed with PBS buffer and incubated with primary antibodies (150 μL of the affinity-purified first antibodies were mixed with 10 mL of the blocking solution) for 2 h at room temperature. After incubation with primary antibodies, bound antibodies were detected using a rat IgG ABC (avidin:biotinylated enzyme complex) kit (Vectastain Elite, Vector Laboratories) according to the manufacturer's protocol. Detection of the amplified signal was carried out with a substrate kit (VIP, Vector Laboratories) according to the manufacturer's protocol. The stained sections were washed with distilled water for 5 min, and then dehydrated and mounted in nonaqueous mounting medium using standard protocols. Slides were examined on a microscope (Labophot-2, Nikon) using bright-field optics.

Plant Transformation

The *MdMADS2* cDNA was cloned into the expression vector pGA1530, a binary vector containing the 35S promoter and T7 terminator, and the *npt (neomycin phosphotransferase)* gene as a selective maker (An et al., 1988). *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) was used for transformation of tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1 and cv Xanthi) plants. Transgenic plants were maintained in greenhouse conditions.

Polyamine Determination

Polyamines were extracted from the tobacco leaves, dansylated, solvent purified, separated by TLC, and quantified with a fluorometer according to the method of Goren et al. (1982). The level of soluble protein was measured by the method of Bradford (1976) using BSA as a standard.

RESULTS

Sequence Analysis of the *MdMADS2* cDNA

The *MdMADS2* cDNA clone is 1230 bp long and contains an ORF of 256 amino acid residues with a 233-bp 5′ leader region and a 229-bp 3′ UTR (accession no. U78948). Alignment of the deduced amino acid sequence of MdMADS2 and other MADS proteins is represented in Figure 1. The MdMADS2 protein has the MADS domain located between amino acid residues 2 and 56 and the K domain located between amino acid residues 93 and 158. The highest overall amino acid identity (76%) was shown by the sequence from silver birch BpMADS5 (accession no. X99655). The

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A
MdMADS2    2 GRGRVQLKRIENKINRQVTFSKRRSGLMKKAHEISVLCDAEVALIIFSTKGKLFEY
BpMADS5    2 ****************************L*****************V**********
TM4        2 ****************************L*************G**V**********
POTM1-1    2 ****************************L*************G**V**********
SLM5       2 ************************T**L************D*G**V**********
AGL8       2 ****************************L*****************V**S*******
SaMADSB    2 ****************************L*****************V***S*******
SQUA       2 ***K*********************G**L*****L**********V**N*******
AP1        2 ************************A**L*****************VV**H*******
Boi2AP1    2 ************************A*******************VV**H*******

B
MdMADS2   93 LEHAKLKARVEVLQRNQRHYMGEDLQSLSLKELQNLEQQLDSALKHIRSRKNQVMYESISELQKK
BpMADS5   93 *********I*******K*FV****D*************************L*********R*
TM4       93 ***R*****L*******K**V****E***M*******H***************L*H****V****
POTM1-1   93 *********L*******K**V****E**NM*******H***************L*H****V***Q
SLM5      93 *********L*I**K*H********DT********F*H***T*******K***L*****H*****
AGL8      93 *************EK*K*NF*****D********S**H***A*I*S*******A*F****A****
SaMADSB   93 *************EK*K*NF*****D********S**H**HA*I*S*******A*F****A****
SQUA      93 **YS*****I*L***H********D*M****I*S******T***N**T****LL*D******H*
AP1       93 M*YNR***KI*L*E******L*****AM*P**********T******T****L*****N*****
Boi2AP1   93 M*YNR***KI*L*E******L*****AM*P**********T**********L**D**N***R*

C                                  Overall identity(%)
MdMADS2   236 PTPHRPNMLLPAWIVRHLNE        100
BpMADS5   225 TP***A*A***P*ML****Q          76
TM4       208 DNGKWKEVV*HSSK*QLIIL          68
POTM1-1   231 QQGAAN*TVM*Q*ML****G          68
SLM5      238 NSSNNNSLVPSWMLNHLAEQ          65
AGL8      223 T*TNELTEPNSLLPAWM*RP          63
SaMADSB   222 T*NESLAEPNSLLPAWM*RP          62
SQUA      229 NELDLTLDS*YSCHLGCFAA          60
AP1       236 NDLELTLEPVYNCNLGCFAA          60
Boi2AP1   237 NDLDLSLEPVYNCNLGCFAA          60
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Figure 1. Comparison of the amino acid sequence of the MdMADS2 protein with other MADS proteins in the SQUA subfamily. A, Sequence alignment of the MADS domain. Shown here are the MADS-box sequences of MdMADS2 (accession no. U78948), silver birch BpMADS5 (accession no. X99655), tomato TM4 (accession no. X60757), potato PTOM1-1 (accession no. U23757), white campion SLM5 (accession no. X80492), Arabidopsis AGL8 (accession no. U33473), white mustard SaMADSB (accession no. U25695), snapdragon SQUA (accession no. X63701), Arabidopsis AP1 (accession no. S35631), and broccoli Bio2AP1 (accession no. U67452). The asterisks represent amino acid residues identical to the corresponding residues in MdMADS2. The numbers at the left represent the positions of the first amino acid residues shown for each sequence. B, Alignment of the K domains. The asterisks and the numbers at the left are as represented in A. C, Alignment of the C-terminal regions. The last 20 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 identical amino acid residues with MdMADS2.

MADS domain shares an identity of 96%. The MdMADS2 protein shows over 60% overall amino acid identity to TM4 from tomato (Pnueli et al., 1991), PTOM1-1 from potato (Kang and Hannapel, 1995), SLM5 from white campion (Hardenack et al., 1994), AGL8/FRUITFULL from Arabidopsis (Mandel and Yanofsky, 1995b; Gu et al., 1998), SaMADS B from white mustard (Menzel et al., 1996), SQUA from snapdragon (Huijser et al., 1992), AP1 from Arabidopsis (Mandel et al., 1992), and Boi2AP1 from broccoli (Carr and Irish, 1997), which are the SQUA subfamily members (Theissen et al., 1996). In the MADS domain there are more significant similarities (over 90% amino acid identity) between MdMADS2 and the SQUA subfamily members.

Sequence comparison of the MADS family genes showed that the 3′ portion of the genes is the most divergent. A 548-bp cDNA fragment (nucleotides 682–1230) of the *MdMADS2* cDNA clone was used as a probe to determine whether the fragment is gene-specific. Genomic DNA blot analysis revealed that one *Hin*dIII, one *Bam*HI, and one *Pst*I fragment hybridized with the probe, indicating that the 3′ region is gene specific (data not shown). To avoid cross-hybridization, therefore, the cDNA fragment containing only the 3′ region of the gene was used as a probe for analysis of the expression pattern of *MdMADS*2.

RNA Blot Analysis

Flower bud formation in apple trees occurs in the previous growing season. The morphological differentiation proceeds until dormancy occurs in winter. In spring, differentiation resumes and rapid development takes place prior to anthesis.

The mRNA expression pattern of *MdMADS2* was examined in flower buds that resumed growth in the spring (March–May). The flower buds were sampled on the basis of the bud length and morphological events, as previously described (Sung and An, 1997). As shown in Figure 2, the *MdMADS2* mRNA was not detectable in mature leaves (lane 1), whereas the transcript was detected in flower buds at all five stages of flower development (lanes 2–6). The intensity of the RNA band was strongest at stage 1 (lane 2) and receded as the flower developed. In mature flowers the expression was relatively strong in sepals and petals but weak in stamens and carpels (lanes 7–10).

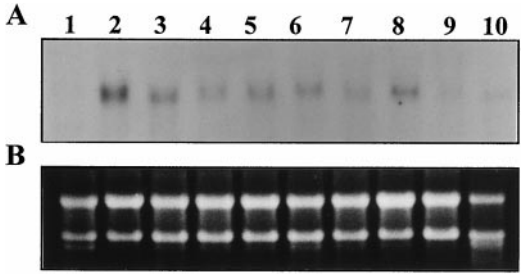

Figure 2. A, RNA blot analysis of the *MdMADS2* transcript in apple flower bud. Lane 1, Mature leaves; lane 2, stage 1 bud (length = 4–5 mm); lane 3, stage 2 bud (length = 7–8 mm); lane 4, stage 3 bud (petals begin to emerge from sepals); lane 5, mature flowers; lane 6, post-anthesis flowers; lane 7, sepals; lane 8, petals; lane 9, stamens; and lane 10, carpels of mature flower. B, rRNA stained with ethidium bromide showing the equivalence of RNA loading between the lines.

RNA in Situ Localization

The temporal and spatial expression patterns of the *MdMADS2* transcript were determined by RNA in situ hybridization. A DIG-labeled *MdMADS2* antisense RNA probe was used. The MADS domain and the K domain were not included to avoid cross-hybridization with other *MdMADS* genes.

Apple has a determinate inflorescence with a terminal flower and a tendency toward dichasial branching (Pratt, 1988). An early stage of apple flower development occurs during the previous growing season. The development of the flower buds at an early stage can be divided into three stages: evocation of the inflorescence meristem (stage 1), differentiation into flower primordia (stage 2), and sequential initiation of sepal, petal, stamen, and carpel primordia in the floral meristem (stage 3). At stage 1, the *MdMADS2* transcript is present throughout the inflorescence meristem, the bud procambium, and the adjacent leaf appendages, but is more concentrated in the early floral meristem arising from the inflorescence meristem (Fig. 3A). At stage 2, *MdMADS2* continues to be expressed in all parts of the flower bud, including flower primordia, bracts, and leaf appendages (Fig. 3B). The signal is high in the floral meristem (Fig. 3B). At early stage 3, when the sepal and petal primordia arise and differentiate, the signal is detected at a high level in the region of the floral meristem interior to the sepal and petal primordia, but at a low level in the sepal and petal primordia (Fig. 3C). At late stage 3, when differentiation of the four floral organ primordia becomes apparent, *MdMADS2* is expressed weakly throughout the sepal, petal, carpel, and stamen primordia (Fig. 3D).

In spring the morphological differentiation of stamens and carpels is accelerated, which is the critical stage in the development of apple flower buds (stage 4). At this stage, stamens develop into anthers and filaments, and the individual carpel fuse with each other to form pistils. At stage 4, *MdMADS2* RNA is uniformly expressed at low levels throughout flower, such as the perianth (sepals and petals), the reproductive organs (stamens and fused carpels), and the floral tube (Fig. 3E). As floral organs mature, the overall hybridization signal becomes weaker (Fig. 3F). In situ expression of *MdMADS2* is detected in all four floral organs of the mature flower, which we have shown by the RNA blot analysis. Inflorescence meristem hybridized with the *MdMADS2* sense probe showed no signal above background (Fig. 3G). We also observed that the sense probe did not show any significant hybridization signal to the sections from the stage of stamen and carpel development and the stage of mature flower development (Fig. 3, H and I).

Immunolocalization

To study the expression pattern of MdMADS2 at a protein level, polyclonal antibodies were raised against a trun-

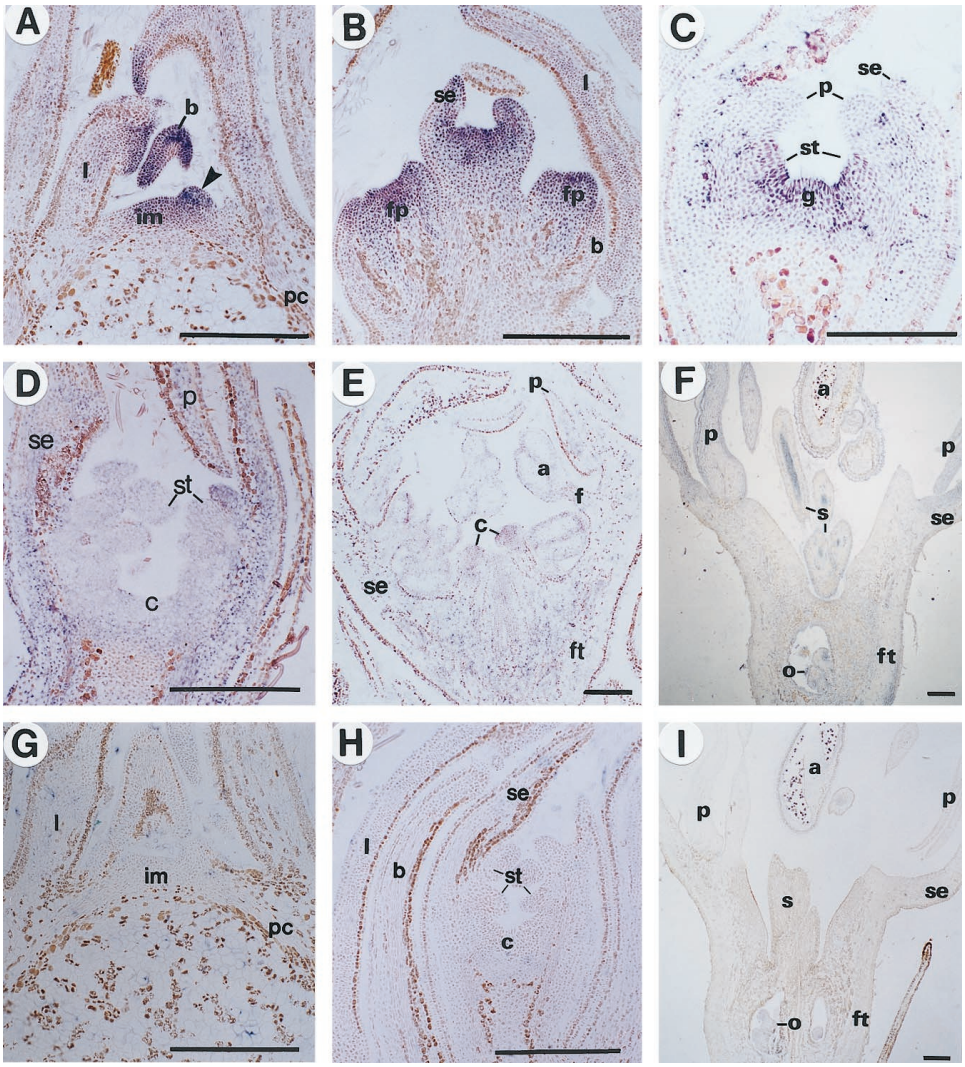

Figure 3. In situ hybridization patterns of *MdMADS2* mRNA in a developing flower bud. Each section was photographed using a blue filter under bright-field optics. The transcript signal is blue. Bar = 300 μm. A, Stage 1 flower bud with the inflorescence meristem. The arrowhead indicates the early floral meristem emerging from the inflorescence meristem. B, Stage 2 flower bud with young flower primordia. C and D, Flower buds at early and late stage 3, respectively. Floral organs arise and differentiate at these stages. E and F, Stage 4 flower bud with fused carpels and mature flower, respectively. G through I, Hybridization with a sense probe of *MdMADS2* in flower bud at stage 1 (G), stage 3 (H), and stage 4 (I). a, Anther; b, bract; c, carpel; f, filament; fp, flower primordium; ft, floral tube; g, gynoecium; im, inflorescence meristem; l, leaf appendage; o, ovule; p, petal; pc, procambium; s, style; se, sepal; st, stamen.

cated form of MdMADS2 lacking the conserved MADS domain. The primary antibodies were affinity purified with antigen and used for further studies. Protein blot analysis was conducted to determine whether the antibodies cross-reacted with other MADS proteins, with OsMADS1 from rice (Chung et al., 1994), or with MdMADS3 and MdMADS4 from apple (S.-K. Sung, G.-H. Yu, and G. An, unpublished data). In all cases, the truncated proteins lacking the MADS domain were expressed in *E. coli*. As shown in Figure 4, the antibodies reacted only with MdMADS2 and did not recognize other MADS proteins. We also tested cross-reactivity with other antibodies prepared against the truncated MdMADS3, MdMADS4, and OsMADS1 proteins. These antibodies immunoreacted with their own proteins, but did not recognize the MdMADS2 protein (data not shown). Therefore, the MdMADS2 antibodies generated in this study are specific for detection of MdMADS2.

It is possible that the transcription pattern of *MdMADS2* does not reflect its protein level if the gene is regulated at the posttranscriptional level. Therefore, the expression pattern of the MdMADS2 protein was examined by in situ immunolocalization. At stage 1, MdMADS2 protein is seen in the inflorescence meristem, the bud procambium, and the adjacent leaf appendages in the flower bud (Fig. 5A), which is consistent with the results of *MdMADS2* mRNA in situ hybridization shown in Figure 3A. In the bud with the vegetative apex (leaf bud), the protein signal was not detected in the apical stem meristem, bud procambium, or the

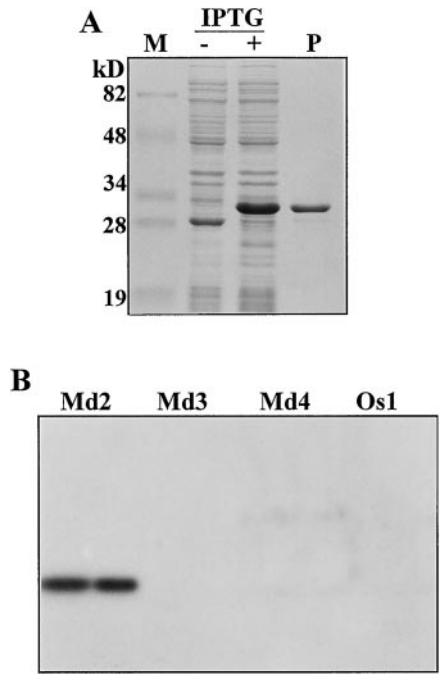

Figure 4. MdMADS2 protein expressed in *E. coli* and protein gel blots of MdMADS2 and other MADS proteins with affinity-purified anti-MdMADS2 antibodies. A, Coomassie Blue-stained protein gel showing truncated MdMADS2 protein expressed in *E. coli*. Lane M, M_r marker; lane −, before IPTG induction; lane +, after IPTG induction; lane P, purified MdMADS2 protein. B, Protein gel blots of truncated MADS proteins with affinity-purified anti-MdMADS2 antibodies. Each protein was loaded in two adjacent lanes. Md2, MdMADS2; Md3, MdMADS3; Md4, MdMADS4; Os1, OsMADS1.

adjacent leaf appendages (Fig. 5C). Expression of MdMADS2 continues throughout the young flower primordia and the emerging sepal primordia by stage 2 (Fig. 5E).

The MdMADS2 protein was clearly localized to the nucleus (data not shown). When differentiation of the four floral organ primordia became apparent (stage 3), the MdMADS2 protein was highly expressed in developing sepals and petals, but was hardly detectable in the regions of the emerging stamen and carpel primordia (Fig. 5G). Considering the RNA in situ hybridization result as shown in Figure 3, C and D, which shows a significant expression of *MdMADS2* mRNA in stamen and carpel primordia, it is likely that posttranscriptional regulation is involved in controlling MdMADS2 expression in the sites of stamen and carpel primordia. During this stage, the rib meristem is very active in the formation of the elongated receptacle, and a higher level of MdMADS2 expression was observed. The protein signal was also detected in bracts and leaf appendages. Only low background was detected in control experiments utilizing preimmune serum as the primary antibody, demonstrating that the signals observed with the MdMADS2 antibody are specific (Fig. 5, B, D, F and H).

Ectopic Expression in Tobacco

We used a transgenic approach to study the influence of expression of *MdMADS2* on the development of the plants. Because it is a long procedure to generate and analyze transgenic apple trees and their flowers, we employed a heterologous tobacco system. The *MdMADS2* cDNA was placed under the control of the CaMV 35S promoter and the chimeric molecule was introduced to the tobacco genome by *A. tumefaciens* co-cultivation. More than 20 independent transformants were regenerated from two culti-

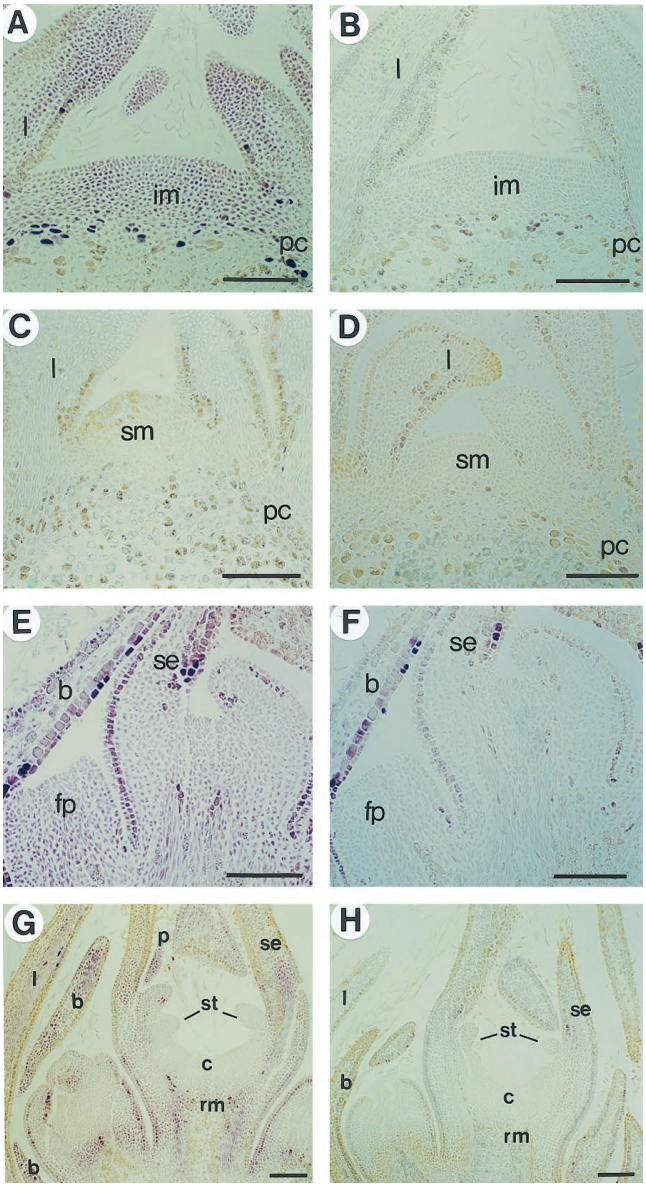

Figure 5. In situ immunolocalization of the MdMADS2 protein in a developing flower bud. Sections were hybridized with affinity-purified anti-MdMADS2 antibodies. Sections were photographed using a blue filter under bright-field optics. The protein signal is purple. Bar = 100 μm. A, Flower bud with the inflorescence meristem (stage 1). B, Preimmune serum control for A. C, Vegetative bud with the stem meristem. D, Preimmune serum control for C. E, Flower bud at stage 2. F, Preimmune serum control for E. G, Flower bud at stage 3. H, Preimmune serum control for G. b, Bract; c, carpel primordium; im, inflorescence meristem; fp, flower primordium; l, leaf appendage; p, petal primordium; pc, procambium; rm, rib meristem; se, sepal primordium; sm, stem meristem; st, stamen primordium.

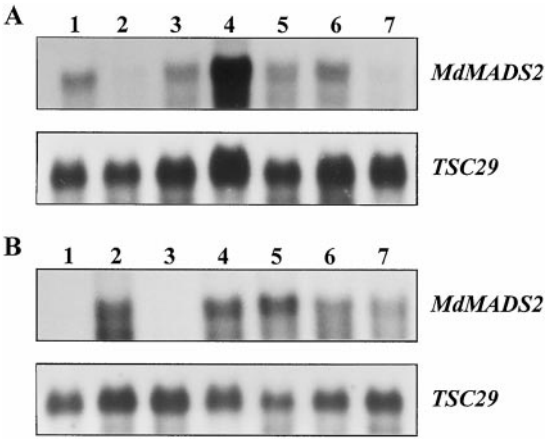

Figure 6. RNA blot analysis of primary transgenic tobacco plants. The numbers indicate independent transgenic tobacco lines of two different cultivars, SR1 (A) and Xanthi (B). Twenty micrograms of total RNA was isolated from mature leaves and hybridized with the ^{32}P-labeled *MdMADS2* probe. As a control, a probe prepared from the *TSC29* cDNA (Gao et al., 1994) was hybridized to the same membrane after washing off the *MdMADS2* probe.

vars, SR1 and Xanthi. Five lines that showed a dwarf phenotype and two lines with weak phenotypes were selected from each cultivar for further analysis. The five lines that showed the severe dwarf phenotype had significantly higher *MdMADS2* expression levels than those of the weak phenotype plants (Fig. 6). The SR1 line 4 and Xanthi lines 4 and 5 that showed a high level of transgene expression and the severe dwarf phenotype were self-pollinated and phenotypes of offspring were analyzed. The dwarf phenotype was inherited in the next generation as a dominant Mendelian trait and co-segregated with the kanamycin-resistance gene (data not shown).

The phenotypes of the homozygous progeny are described in Table I. The morphological changes in transgenic plants were not seen until the appearance of several leaves (data not shown). At flowering time, the plant height of the SR1 line 4 was reduced by an average of 30 cm and that of

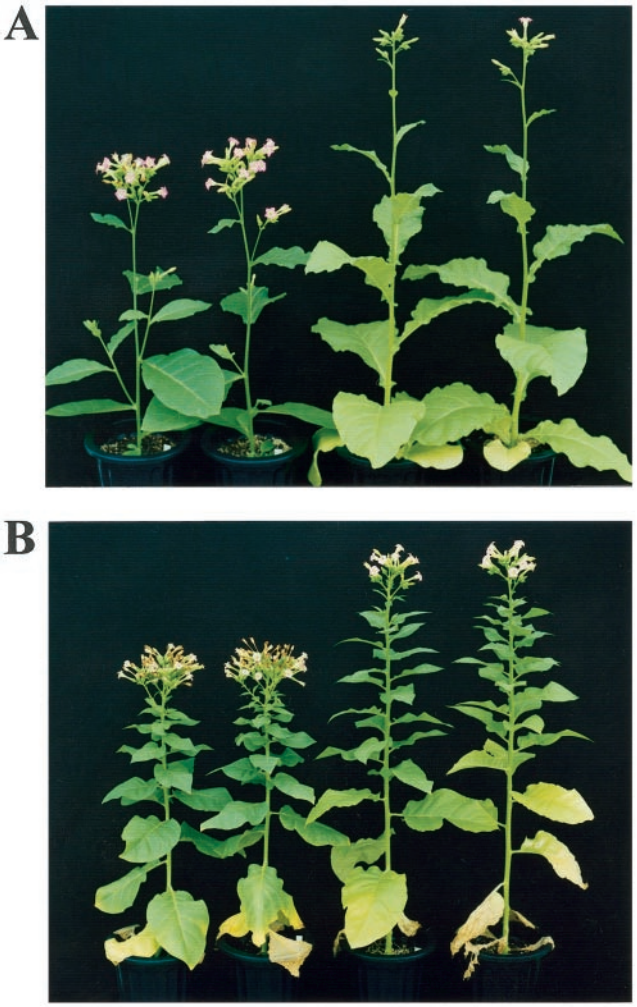

Figure 7. Ectopic phenotypes of transgenic tobacco plant. A, Homozygous transgenic line S4 (left two plants) and wild type (right two plants) of the tobacco cv SR1. B, Homozygous transgenic line X4 (left two plants) and wild type (right two plants) of the tobacco cv Xanthi.

Table I. *Phenotypes of transgenic tobacco plants*

The homozygous progeny (T_2 generation) from the primary transformed line (T_1 generation) were germinated in a peat pellet and maintained in greenhouse conditions. Progeny carrying the transgenes were identified by visually scoring T_3 seedlings for kanamycin resistance. Wild-type tobacco plants were used as controls. Each value represents the mean ± SD (n = 10). The experiment was repeated three times with similar results.

Transgenic Line	Days to Flowering[a]	Height[b]	Nodes[c]	Contents of Polyamines[d]
		cm	*n*	*μg mg^{-1} protein*
cv SR1				
Wild type	66.4 ± 0.9	85.0 ± 5.7	16.0 ± 1.3	3.7 ± 0.9
Transgenic S4	56.2 ± 1.0	55.3 ± 4.3	8.0 ± 0.7	14.5 ± 1.7
cv Xanthi				
Wild type	86.3 ± 3.8	88.0 ± 2.2	39.0 ± 2.1	9.3 ± 2.2
Transgenic X4	78.2 ± 2.6	75.4 ± 5.1	28.0 ± 1.7	12.6 ± 2.9

[a] Days to flowering is defined as the time from seed sowing to the time when the first petals opened. [b] Height and [c] number of nodes were measured when fruits were fully developed. [d] Contents of polyamines (putrescine + spermidine + spermine) were measured in the fifth and sixth leaves of plants.

Xanthi line 4 was reduced by 13 cm (Fig. 7). This reduction seemed to coincide with the decrease in node number; the SR1 line 4 and Xanthi line 4 produced one-half to two-thirds the number of nodes produced by wild-type plants (Table I). The node length was similar to that of wild-type plants. We observed that the number of days to flowering time was shortened by 8 to 10 d in transgenic plants. Furthermore, floral development occurred at the axillary buds positioned at lower nodes, which give rise to leaf primordia in wild-type plants. Therefore, it appears that overexpression of the *MdMADS2* gene promoted the commitment of indeterminate meristems to flower and at the same time arrested the vegetative growth. Other phenotypic alterations in vegetative parts include smaller and greener leaves. There was no visible alteration in floral organ morphology. When immunolocalization assays were performed MdMADS2 protein accumulated in transgenic plants (data not shown), whereas there was no MdMADS2 protein signal in wild-type plants (data not shown).

Polyamine is known as a growth substance involved in apple flower bud development. We therefore examined polyamine (putrescine + spermidine + spermine) contents in transgenic plants. Transgenic plants exhibited elevated levels of the polyamine contents in leaves that were 1.5- to 4-fold over those of the wild type (Table I).

DISCUSSION

The MADS genes are components of complex networks of genes that play an important role in flower development and are found in a variety of plant species. We report the isolation and characterization of a new MADS-box gene from Fuji apple, *MdMADS2*. A multiple alignment of MdMADS2 with other MADS proteins revealed that MdMADS2 showed high sequence relatedness to various MADS proteins in the SQUA subfamily (Theissen et al., 1996), which includes TM4 from tomato (Pnueli et al., 1991), PTOM1-1 from potato (Kang and Hannapel, 1995), SLM5 from white campion (Hardenack et al., 1994), AGL8/FRUITFULL from Arabidopsis (Mandel and Yanofsky, 1995b; Gu et al., 1998), SaMADS B from white mustard (Menzel et al., 1996), SQUA from snapdragon (Huijser et al., 1992), AP1 from Arabidopsis (Mandel et al., 1992), and Boi2AP1 from broccoli (Carr and Irish, 1997). MdMADS2 shares over 60% overall amino acid identity with the product of these genes and the functional MADS domain shows over 90% amino acid identity.

The most fully characterized MADS genes in the *SQUA* subfamily are *SQUA* of snapdragon and *AP1* of Arabidopsis, which control floral meristem identity (Huijser et al., 1992; Mandel et al., 1992). *SQUA* and *AP1* are expressed in floral meristems as soon as they form on the flanks of the inflorescence meristem (Huijser et al., 1992; Mandel et al., 1992). In contrast, *AGL8/FRUITFULL*, which diverged from *AP1* via gene duplication of Arabidopsis, is expressed in the inflorescence meristem but not in the floral meristem, suggesting that it may be involved in the inflorescence meristem identity (Mandel and Yanofsky, 1995b; Purugganan et al., 1995). A loss-of-function analysis revealed further that the *AGL8/FRUITFULL* gene is required for cellular differentiation during fruit and leaf development (Gu et al., 1998).

Unlike *SQUA*, *AP1*, and *AGL8/FRUITFULL*, *MdMASDS2* is expressed in both the inflorescence meristem and floral meristems in apple flower buds. Expression of *SLM5*, a *SQUA* homolog from white campion, has also been detected both in the inflorescence and and in the floral meristems (Hardenack et al., 1994). It has been suggested that the expression of *SLM5* both in inflorescence meristems and floral meristems in white campion may reflect the structure of the inflorescence (Hardenack et al., 1994). White campion has a determinate dichasial inflorescence in which the apical meristem forms a flower flanked by two lateral inflorescence meristems, whereas snapdragon and Arabidopsis have an indeterminate apical inflorescence meristem from which flowers develop on the flanks. Apple has a determinate inflorescence with a terminal flower and a tendency toward dichasial branching (Pratt, 1988). Thus, the determinate inflorescence of apple could result in expression of *MdMADS2* in both the inflorescence and the floral meristems.

In subsequent stages, the *MdMADS2* transcript is present in all of the floral organs and organ primordia, including the stamen. We also observed *MdMADS2* expression in individually dissected stamens and the other floral organs on northern blots. This feature was unexpected given the expression patterns of *SQUA*, *AP1*, *AGL8/FRUITFULL*, and *SLM5*; these genes are not expressed in stamens at any developmental stage (Mandel et al., 1992; Hardenack et al., 1994; Kempin et al., 1995; Mandel and Yanofsky, 1995b). In contrast to these genes, *BoiAP1* from broccoli, a member of the Brassicaceae, is expressed in the stamen primordia (Carr and Irish, 1997). Moreover, expression of *TM4*, a *SQUA* homolog of tomato, is observed in all of the floral organs when plants are grown at low temperature (Lozano et al., 1998).

At standard temperatures, transcripts of *TM4* are not detected in total RNA from any floral organ (Pnueli et al., 1991). Regulation of homeotic gene expression by hormonal or environmental factors has been suggested in several studies (Estruch et al., 1993; Okamuro et al., 1996; Venglat and Sawhney, 1996). It is generally accepted that the development of apple flower buds is the process most sensitive to hormonal, nutritional, and environmental factors, because induction and development of flower buds occur on the trees carrying a heavy crop of fruit (Buban and Faust, 1982; Pratt, 1988). Therefore, it is possible that expression patterns of *MdMADS2* in apple are not only affected by growth pattern, such as the structure of the inflorescence, but are also somehow affected by hormonal, nutritional, and environmental factors.

The diversity in the expression patterns of *MdMADS2* and other genes in the *SQUA* subfamily could result from their divergent functions. If the genes are regulated at posttranscriptional level, however, the transcription patterns may not reflect the region of the plant where the protein is present. Therefore, we examined the expression pattern of the MdMADS2 protein. At early developmental stages of the floral meristem, the accumulation patterns of the protein were generally in agreement with those of the

MdMADS2 mRNA. However, we observed a discrepancy between the transcript level and the protein level after stamen and carpel differentiation. The MdMADS2 protein was not detectable in the stamen and carpel primordia, where a significant RNA signal was detected.

These results indicate the possibility that a posttranscriptional regulatory mechanism may be involved in the *MdMADS2* expression in stamen and carpel primordia. Posttranscriptional regulation of plant gene expression is not uncommon. In situ experiments of the petunia *fbp1* (*floral binding protein*) gene demonstrated that *fbp1* gene expression is posttranscriptionally regulated in stamen primordia at later stages of development (Canas et al., 1994). Furthermore, posttranscriptional regulation of the *AP3* gene has been shown in transgenic Arabidopsis plants ectopically expressing *AP3*. *AP3* mRNA was detected throughout the inflorescence and vegetative tissues, but the AP3 protein was only detected in the second, third, and fourth floral whorls (Jack et al., 1994). Krizek and Meyerowitz (1996) proposed that the posttranscriptional regulation of *AP3* is modulated by the formation of a heterodimer with the PI protein.

Although we were unable to test the functions of the apple *MdMADS2* by mutation analysis, it is possible to predict the putative functions of the gene by analysis of its expression patterns. The expression patterns of *MdMADS2* at the RNA and protein levels suggest that *MdMADS2* is active in inflorescence and floral meristems at early developmental stages and its activity is modulated in stamen and carpel primordia by posttranscriptional regulation.

Ectopic expression is a useful method with which to analyze the influence of expression of genes that regulate developmental processes. Transgenic tobacco plants ectopically expressing *MdMADS2* exhibited early flowering and development of auxiliary flowers. There was no visible change in the floral organs. Mandel and Yanofsky (1995a) demonstrated that ectopic expression of *AP1* is not only sufficient to convert apical and lateral shoots into flowers, but also to cause an early flowering phenotype in Arabidopsis. Several other MADS-box genes have been shown to promote early flowering when overexpressed (Chung et al., 1994; Kyozuka et al., 1997; Kater et al., 1998; Tandre et al., 1998). In addition to being involved in early flowering, ectopic expression of *MdMADS2* is involved in production of smaller and greener leaves and increase in the polyamine content in transgenic plants. It is generally believed that polyamines function as growth substances and promote flower development in higher plants (Smith, 1985). Polyamines are also involved in morphogenesis (Evans and Malmberg, 1989) and bud break in perennial trees (Wang and Faust, 1994).

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