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Cloning and characterization of four apple *MADS* box genes isolated from vegetative tissue

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Abstract

With the aim of finding genes involved in the floral transition of woody species four *MADS* box genes containing cDNAs from apple (*Malus domestica*) have been isolated. Three genes were isolated from vegetative tissue of apple, but were homologues of known genes that specify floral organ identity. *MdMADS13* is an *AP3*-like B class *MADS* box gene, and was mainly expressed in petals and stamens as demonstrated by Northern blot analysis. *MdMADS14* and *–15* are *AGAMOUS*-like genes. They differed slightly in expression patterns on Northern blots, with *MdMADS15* mRNA levels equally high in stamens and carpels, but *MdMADS14* preferably expressed in carpels. *MdMADS14* is likely to be the apple orthologue of one of the *Arabidopsis thaliana SHATTERPROOF* genes, and *MdMADS15* closely resembled the *Arabidopsis AGAMOUS* gene. It has been shown with RT-PCR that the three floral apple *MADS* box genes are expressed in vegetative tissues of adult as well as juvenile trees, albeit at low levels. *MdMADS12* is an *AP1*-like gene that is expressed at similar levels in leaves, vegetative shoots, and floral tissues, and that may be involved in the transition from the juvenile to the adult stage.

Key words: Apple, flower development, *MADS* box, floral transition.

Introduction

Understanding the processes regulating the phase transition from vegetative to reproductive growth is particularly important for woody fruit crops, like apple (*Malus domestica*). Apple has a long juvenile phase of 4–8 years, which means that breeding programmes for favourable traits related to fruit production and fruit quality take a long time. Knowledge of the genes governing the transition from the juvenile to the adult stage during which reproductive growth is initiated may facilitate selection of plants with shortened juvenile phases, and may enable interference in the processes governing this transition.

It is very likely that at least some of the genes signalling the end of the vegetative phase and the beginning of the reproductive phase are members of the *MADS* box gene family. *MADS* box genes are mostly regulators of developmental processes (Shore and Sharrocks, 1995). *MADS* box genes in plants were originally identified as homeotic genes with important roles in flower development. Extensive studies in *Arabidopsis thaliana*, *Antirrhinum majus*, *Petunia hybrida*, and recently also in a number of other species, have revealed that specific *MADS* box genes, alone or in combination, serve as regulators for the formation of the flower organs (Weigel, 1995; Theissen and Saedler, 1999; Theissen *et al*., 2000). These genes fit the ABC model of floral organ identity, originally based on studies of homeotic flower mutants in *Arabidopsis* and *Antirrhinum* (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). The A function defines sepal formation and depends on expression of the *MADS* box gene *APETALA1* in *Arabidopsis*. Petal identity is defined by A-type as well as B-type gene expression (*MADS* box genes *APETALA3*/*PISTILLATA* in *Arabidopsis*, *DEFICIENS* and *GLOBOSA* in *Antirrhinum*). B-type gene expression in combination with expression of a C-type *MADS* box gene (*AGAMOUS* in *Arabidopsis*, *PLENA* in *Antirrhinum*) is required for

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Abbreviations: 5′RACE, 5′-Rapid Amplification of cDNA Ends; DEPC, diethyl pyrocarbonate; *MdMADS*, *Malus domestica MADS* box; RT-PCR, reverse transcriptase PCR.

development of stamens. Carpel formation is dependent on C-type *MADS* box gene expression alone. The roles of *MADS* box genes in flower development are well conserved in all angiosperms, including monocotyledonous and dicotyledonous species, and some specific functions of *MADS* box genes are retained even in gymnosperms (Purugganan *et al*., 1995, 1997; Theissen *et al*., 1996, 2000).

Recent studies demonstrated that the functions of *MADS* box gene family members are not restricted to flower organ development. *MADS* box genes were shown to be involved in initiation of flowering, determination of meristem identity (Weigel, 1995), embryonic development (Perry *et al*., 1999), root formation (Alvarez-Buylla *et al*., 2000), development of vascular tissue, and seed and fruit formation (Buchner and Boutin, 1998; Gu *et al*., 1998; Liljegren *et al*., 1998). Furthermore, a single *MADS* box gene may have different functions at different stages of development. The *Arabidopsis* gene *FRUITFULL* (*AGL8*) was suggested to be involved in both determination of meristem identity and, later on, of carpel development (Hempel *et al*., 1997; Gu *et al*., 1998; Ferrandiz *et al*., 2000), and *MdMADS4* of apple was suggested to be important for both flower bud and fruit development (Sung *et al*., 2000).

Recently, a role for *MADS* box genes has been proposed in floral initiation. The *FLF*/*FLC* locus for delayed flowering in *Arabidopsis* was found to encode a MADS box protein, which acts as a supppressor for reproductive development (Sheldon *et al*., 1999; Michaels and Amasino, 1999). Variation in expression of FLC/FLF has a profound effect on flowering time, and *FLC*/*FLF* interacts with genes from other flowering pathways (*LUMINIDEPENDENS*, *FRIGIDA*) (Sheldon *et al*., 1999; Michaels and Amasino, 1999). In contrast, the *PFG* gene of *Petunia* is a *MADS* box gene that is important for inducing rather than inhibiting the floral transition, and the maintenance of the reproductive identity (Immink *et al*., 1999). The mustard gene *SaMADSA* has been suggested to be part of the signal transduction pathway involved in floral transition of the shoot apical meristem (Bonhomme *et al*., 1997, 2000). Both *SaMADSA* and its *Arabidopsis* orthologue *AGL20* were shown to be activated by the gibberellin pathway (Bonhomme *et al*., 2000; Borner *et al*., 2000).

In order to isolate *MADS* box genes that may be related to the juvenile-to-adult transition of woody species, vegetative tissue of five-year-old flowering as well as non-flowering apple trees was used in an RT-PCR approach with degenerate MADS box primers. One of the isolated genes bears resemblance to the *Petunia* gene *PFG*, and *FRUITFULL* of *Arabidopsis*, which play a role in floral transition. Remarkably, three out of four cDNA sequences isolated from vegetative shoots turned out to be apple analogues of *MADS* box genes that have been implicated in the determination of flower whorl identity.

Materials and methods

Plant material

In 1997, leaves, stems and shoots were sampled of *Malus domestica* trees of the cross Bellida × (Prima × D13). Tissues from a number of 5-year-old trees that were flowering in 1997 were pooled (10 plants in a pool) as well as tissues of trees that were not yet flowering that year. Tissues from flowering and non-flowering plants of the cross Prima × Fiesta were sampled in 1997 and collected in flowering and non-flowering pools. Juvenile material consisted of leaves and shoots of 2-year-old plants of several crosses, sampled in 1998.

RNA isolation and analysis

Frozen plant material was pulverized with mortar and pestle in liquid N_2, and approximately 100 mg of powdered tissue was used to isolate total RNA with the RNeasy plant RNA isolation kit (Qiagen). The RNA was precipitated and the pellet was dissolved in diethylpyrocarbonate (DEPC)-treated H_2O. RNA samples (15–30 μg) were subjected to electrophoresis in formaldehyde-containing agarose gels. RNA was blotted to Hybond N (Amersham Pharmacia Biotech) membranes in 20 × SSC using standard Hybond protocols (provided by the manufacturer). Blots were hybridized with gene-specific α^{32}P-dATP labelled cDNA probes (random primed labelling) for 16 h at 42 °C in 1 M NaCl, 10% dextransulphate, 1% sodium dodecyl sulphate (SDS), 10 mM TRIS-HCl pH 7.5, and 100 μg ml^{-1} denatured salmon sperm DNA. Blots were washed to a stringency of 0.1 × SSC, 0.1% SDS at 65 °C, and exposed to X-ray films for several periods of time.

Isolation of MADS *box gene* cDNAs

For the isolation of the *MADS* box cDNA fragments, 1 μg of total RNA was used to synthesize first strand cDNA from the polyA tail with a T(18) primer using the cDNA–for-PCR kit of Clontech. A MADS box specific degenerate primer (MADS5′) complementary to the 5′-end of the MADS box consensus sequence (the LIKRIEN protein motif; sequence: 5′-ACCTCRGCRTCRCARAGSAC-3′) was used to synthesize second strand cDNA in a linear amplification procedure. For this, 1.5 pmol primer was used in 50 μl of reaction volume (0.5 U Goldstar Taq polymerase (Eurogentec), 20 μM dNTPs, 1.5 mM $MgCl_2$) with 5 min 95 °C denaturation, then 30 cycles of 30 s at 95 °C, 100 s annealing at 60 °C, 120 s extension at 72 °C, and a final 10 min extension period. Immediately following the linear amplification, PCR was performed using the MADS5′ and a poly-dT primer. To the reaction mixture, a preheated 48 μl mixture (15 pmol poly-dT primer, 0.5 U Goldstar Taq polymerase, 1.5 mM $MgCl_2$, 100 μM dNTPs) was added, followed by a single step of 2 min 95 °C, 2 min 40 °C, 5 min 72 °C. Then 15 pmol MADS5′ primer was added, and PCR was performed with 30 cycles of 45 s at 95 °C, 100 s at 60 °C, and 120 s at 72 °C. One μl of this PCR product was used in a second PCR with identical conditions, but with a nested degenerate primer (MADS3′R) instead of the MADS5′ primer. MADS3′R is complementary to the consensus sequence of the MADS box 90 bp downstream of the MADS5′ primer (protein motif: VLCDAEV; sequence 5′-GTKCTYTGY-GAYGCYGAGGT-3′). The product of the second PCR was shown to contain a number of fragments ranging from 150–1100 bp. Several products between 300 and 1100 bp were

excised, re-amplified, and cloned into the T/A cloning vector pCR2.1 (Invitrogen). A number of clones were identified by DNA sequencing and BLAST similarity searches with EMBL and Genbank nucleotide databases. The MADS box gene fragments were re-amplified from a first strand cDNA template in a single PCR with a proofreading DNA polymerase to obtain a reliable DNA sequence.

The remaining cDNA sequence of the four *MADS* box genes was obtained using the 5′/3′RACE (Rapid Amplification of cDNA Ends) kit of Roche Diagnostics. Total RNA served as a template in first strand cDNA synthesis with a gene-specific primer. This fragment was tailed with A-residues, and a second PCR involved a poly-dT primer with an anchor sequence and a second gene-specific primer, 5′ of the first gene-specific. The PCR products were isolated from agarose gels, cloned into PCR2.1 (Invitrogen) and the clones were analysed by sequencing.

The names of the four apple *MADS* box genes presented here follow the nomenclature of recently published apple *MADS* box genes (Sung and An, 1997; Yao *et al*., 1999; Sung *et al*., 1999): *MdMADS* (for *Malus domestica MADS* box gene) 12, 13, 14 and 15. (EMBL accession numbers: *MdMADS12*: AJ 320187 (*MdMADS12a*: AJ320188); *MdMADS13*: AJ25116; *MdMADS14*: AJ25117; *MdMADS15*: AJ25118).

Reverse transcriptase (RT)-PCR

One μg of total RNA (isolated from various tissues using the RNeasy plant RNA isolation kit (Qiagen) was used for first strand cDNA synthesis with the cDNA-for-PCR kit of Clontech. The cDNA was synthesized from a poly-dT primer. This cDNA served as a template in PCR (with the KlenTaq Advantage polymerase mix of Clontech) using gene-specific primers, 35 cycles of 30 s 95 °C, 45 s 60 °C, 90 s 72 °C, preceded by 3 min 95 °C, and followed by 10 min at 72 °C. Fragments were of predicted lengths. Control PCR reactions contained the RNA that was used as a template in the cDNA synthesis.

Results

Isolation of Malus domestica *MADS box cDNA*

For the isolation of *Malus domestica* MADS box cDNAs related to the juvenile-to-adult phase change and the transition to flowering, total RNA was isolated from vegetative shoots of both flowering and non-flowering 5-year-old trees of two different crosses. This RNA was used in a RT-PCR procedure with degenerate MADS box primers, starting with asymmetric amplification on first strand cDNA (synthesized from the polyA tail) with the 5′-MADS box primer. This step serves to increase the copy number of MADS box cDNA sequences prior to performing the PCR with this primer and the non-selective oligo-dT primer. It was found that a second PCR with a nested MADS box degenerate primer was necessary to obtain specific products.

The two-step RT-PCR approach yielded a number of bands on agarose gel, ranging in size from 200–1100 bp. Bands between 350 and 1100 bp were subcloned, and found to contain almost exclusively MADS box cDNA fragments. Four different cDNA fragments were identified with similarity to known *MADS* box genes of other species. The shorter fragments of the RT-PCR procedure often represented truncated forms of these sequences. The remaining 5′-sequences of the four cDNAs (including most of the MADS box region) were obtained by 5′-RACE with total RNA of vegetative shoots (*MdMADS12*) and flower buds (*MdMADS13–15*) of adult trees. Sequence analysis revealed that three out of the four cDNA sequences were homologous to B- and C-type *MADS* box genes involved in flower morphogenesis (Fig. 1), even though the starting material was vegetative tissue. The fourth MADS box cDNA resembled genes implicated in the floral transition and determination of floral meristem identity of other species.

Sequence characterization of MdMADS12, -13, -14, *and* -15

MdMADS12 showed highest sequence similarity with the members of the *AP1* group of *MADS* box genes, as depicted in the dendogram of Fig. 1 (see Fig. 2a for alignment with *AP1*-like genes). The genes within this clade are mostly involved in floral transition and early flower meristem development. *MdMADS12* cDNA has the largest open reading frame of 271 amino acids (aa). 5′RACE experiments indicated the possible presence of two major transcription start sites, 62 bp and 207 bp upstream of the ATG startcodon (Fig. 2b). The clones resulting from the two-step RT-PCR approach that represented *MdMADS12* gene sequences could be divided in two groups with identical sequence in most of the coding region, but differing at the 3′-end. *MdMADS12* had a stopcodon 135 bp after the point where the sequences diverge, at 678 bp from the ATG startcodon (aa 226). In *MdMADS12a*, this part of the cDNA was replaced by a repeat-rich sequence, with an in-frame stop codon 63 bp beyond the point where *MdMADS12* and *−12a* sequences diverge (Fig. 2c). Specific primers were used for the two *MdMADS12* sequences on genomic DNA to see whether *MdMADS12* and *MdMADS12a* were the result of alternative splicing. Both *MdMADS12* and *MdMADS12a* sequences were amplified from genomic DNA. The *MdMADS12* coding sequence within this fragment was divided by an intron, with no resemblance to *MdMADS12a* repeat sequence. *MdMADS12a* genomic sequence was identical to the mRNA sequence. This indicates that both *MdMADS12* and *MdMADS12a* were present in the genome as transcriptionally active genes.

The *MdMADS13* full length cDNA is 1102 bp long. The coding region is 699 bp, encoding a 232 amino acid protein (Fig. 3). The 3′-non-coding region is 333 bp, and the 5′-non-coding region 70 bp. *MdMADS13* fits within the clade of B-type *MADS* box genes, more specifically in the *AP3* group (Fig. 1, see Fig. 3 for alignment with other B-type genes). B-type *MADS* box genes are involved in petal and stamen development, and mainly

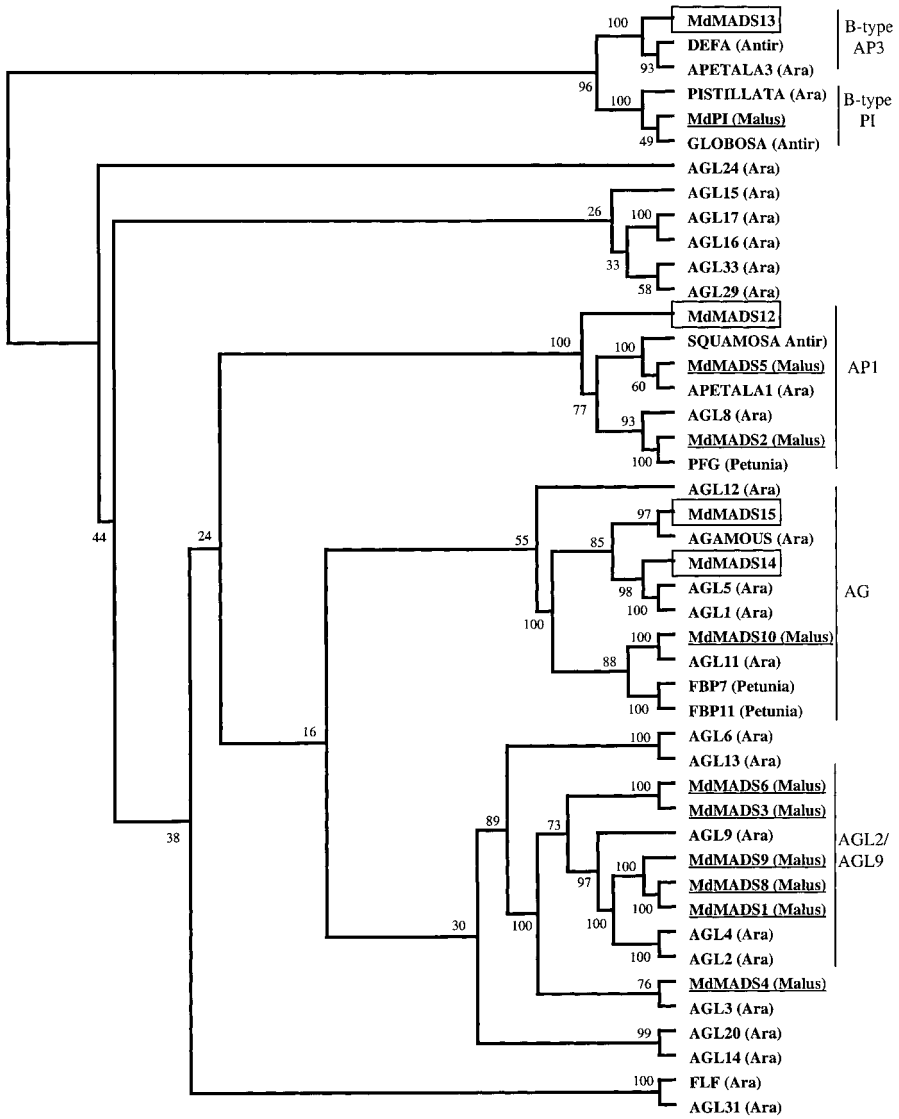

Fig. 1. Phylogenetic tree of apple MADS box protein sequences with known MADS box protein sequences. Complete protein sequences (where necessary translated from the cDNA sequence) were aligned with the ClustalX program (default settings) (latest version at: ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX, website: http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX). The tree was compiled with the Neighbor-Joining method. Values at nodes indicate bootstrap percentages. Boxed apple MADS box protein sequences are translations from the full-length cDNA sequences presented in this paper. Other protein sequences were extracted from the public databases; Apple genes are underlined. Malus is *Malus domestica*, Ara is *Arabidopsis thaliana*, Petunia is *Petunia hybrida*, and Antir is *Antirrhinum major*.

expressed in the second and third whorl of the flower (Weigel and Meyerowitz, 1994). In most species, the B-function is specified by at least two B-type *MADS* box genes. The *PISTILLATA*-like apple gene (*MdPI*) has been published recently (Yao *et al*., 2001), and *MdMADS13* is likely to be the apple *AP3* orthologue.

MdMADS14 cDNA is 1057 bp long, with a largest open reading frame of 729 bp, encoding a protein of 242 amino acids (Fig. 4). 5′- and 3′-non-coding regions are 131 bp and 197 bp, respectively. This cDNA is most similar to the *SHP1* and *SHP2* genes (formerly *AGL1* and *AGL5*) (Ma *et al*., 1991; Liljegren *et al*., 2000), and fits in the *AGAMOUS* clade (Fig. 1, see Fig. 4 for alignment with clade members), suggesting a role primarily in stamen and carpel development (Weigel and Meyerowitz, 1994) and in fruit formation (Liljegren *et al*., 2000). Similar to *AGAMOUS*, the *MADS* box is not located at the N-terminal end of the protein, but is preceded by a short peptide stretch. *MdMADS14* differs significantly from the only other known *AGAMOUS*-like gene of apple, *MdMADS10* (Yao *et al*., 1999) (see alignment of Fig. 5).

MdMADS15 cDNA is 1039 bp long, with a 738 bp open reading frame, coding for a 245 amino acid protein (Fig. 5). The 5′-non-coding region is 70 bp, the 3′-non-coding sequence 231 bp. *MdMADS15* tightly resembled

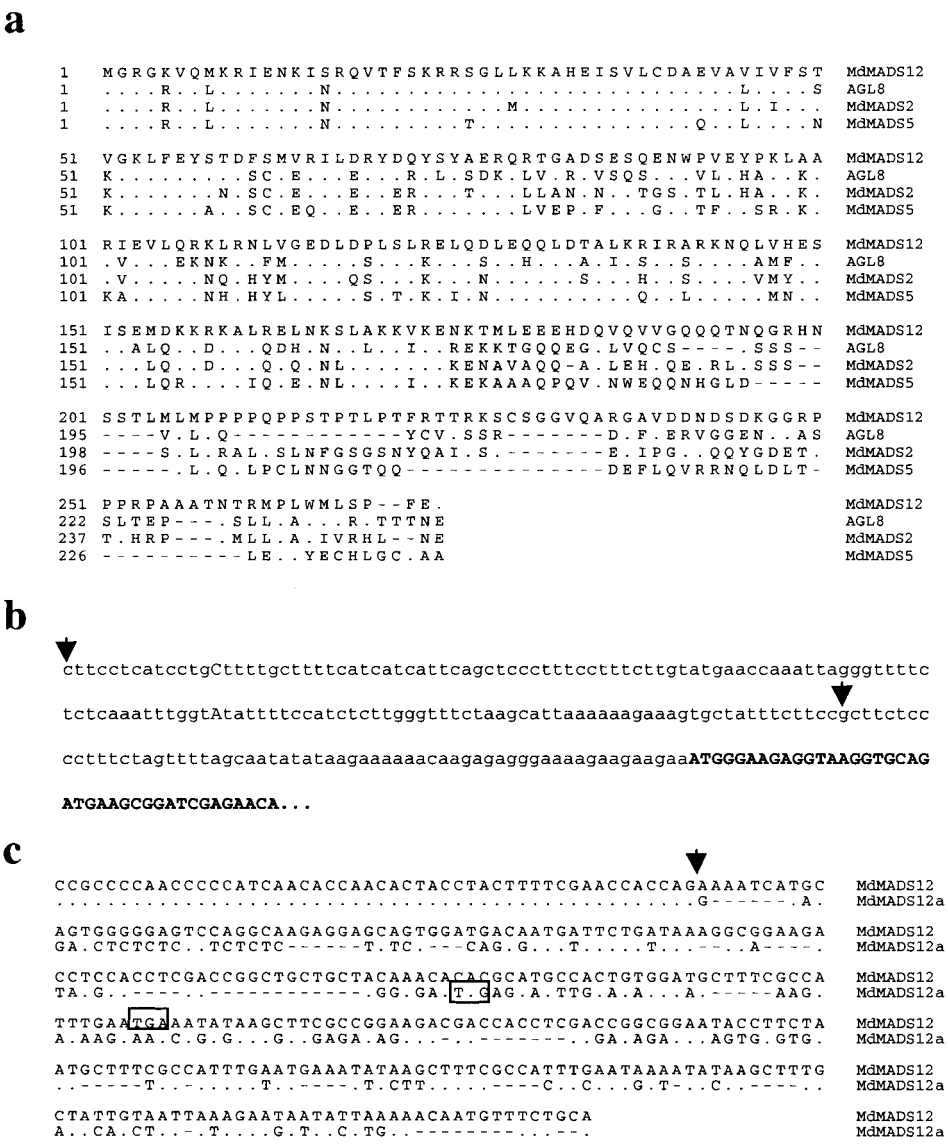

Fig. 2. (a) Deduced amino acid sequence of *MdMADS12* and alignment (CLUSTAL) with *FRUITFULL* (formerly *AGL8* of *Arabidopsis thaliana*, acc. no. U33473), *MdMADS2* (acc. no. U78948) and *MdMADS5* (acc. no. AJ000759) (*Malus domestica*). Dots indicate identical amino acids, dashed lines are gaps. (b) 5′-non-translated region of *MdMADS12*. Arrows indicate putative transcription initiation sites. Non-coding is in lowercase, coding is in uppercase. (c) 3′-part of the cDNAs of *MdMADS12* and *MdMADS12a*. Arrow indicates point from where sequences diverge. Boxes indicate in-frame stopcodons.

MdMADS14 in the *MADS* box to *K* box region, but differs significantly in the 3′-part of the cDNA (Fig. 5).

MdMADS15 is most similar to *AGAMOUS* of *Arabidopsis*, and *AGAMOUS* orthologues of other species (Figs 1, 5), pointing to a function in the development of the reproductive flower organs (Weigel and Meyerowitz, 1994). Highest similarity was observed with *MASAKO C1*, the *AGAMOUS* orthologue of rose, another member of the Rosaceae family (Kitahara and Matsumoto, 2000). As with *MdMADS14* and other *AGAMOUS*-like genes, the *MADS* box is preceded by a short peptide stretch.

mRNA expression analysis of MdMADS12

Northern blot analysis demonstrated that MdMADS12 mRNA was expressed in floral tissue as well as leaves and shoots of adult flowering, adult non-flowering, and juvenile trees (Fig. 6a), with highest expression levels in leaves, and no expression detected in stem material (juvenile nor adult). The signals on the Northern blot indicate that sizes of the transcripts found in vegetative and floral tissue may be slightly different. In tissues where MdMADS12 and MdMADS12a are co-expressed, the signal on the Northern blot may represent expression levels of both MdMADS12 and MdMADS12a: the

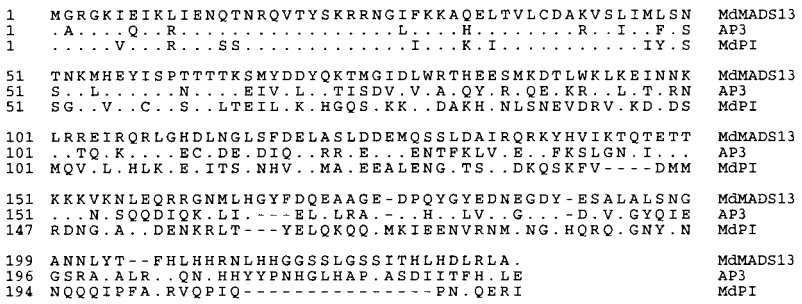

Fig. 3. Deduced amino acid sequence of *MdMADS13* and alignment (CLUSTAL) with *AP3* (*B*-type gene from *Arabidopsis thaliana*, acc. no. M86357) and *MdPI* (*PISTILLATA* orthologue from *Malus domestica*, acc. no. AJ291490). Dots indicate identical amino acids, dashed lines are gaps.

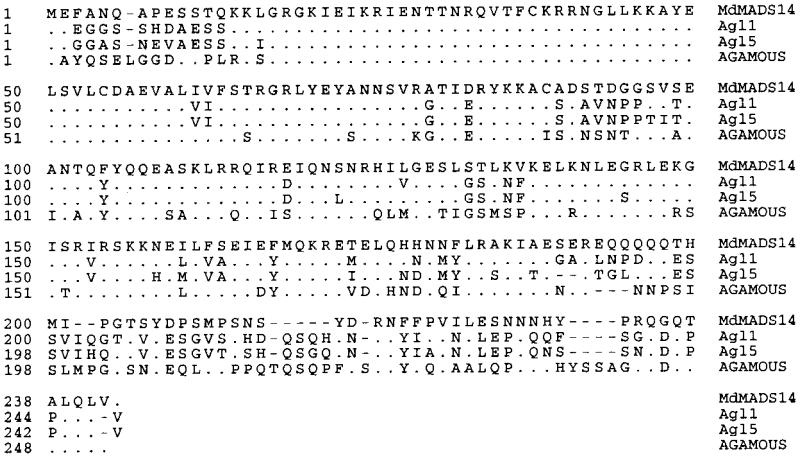

Fig. 4. Deduced amino acid sequence of *MdMADS14* and alignment (CLUSTAL) with *AGL1* (*Arabidopsis thaliana*) (acc. no.: M55550), *AGL5* (*Arabidopsis thaliana*) (acc. no.: M55553) and *AGAMOUS* (*Arabidopsis thaliana*) (acc. no. P17839). Dots indicate identical amino acids, dashed lines are gaps.

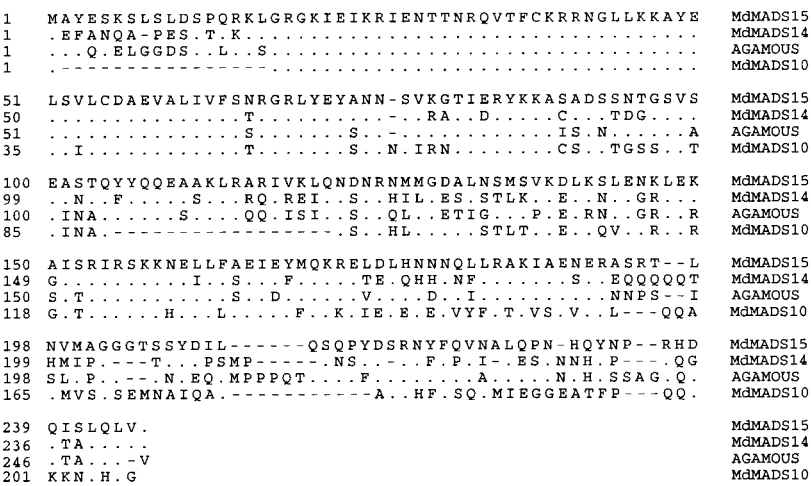

Fig. 5. Deduced amino acid sequence of *MdMADS15* and alignment (CLUSTAL) with *MdMADS14*, *AGAMOUS* (*Arabidopsis thaliana*) (acc. no. P17839) and *MdMADS10* (*Malus domestica*) (acc. no. AJ000762). Dots indicate identical amino acids, dashed lines are gaps.

MdMADS12 probe used for the Northern blot is a MdMADS12 fragment that is for two-thirds identical to MdMADS12a. Using specific primers for each form in RT-PCR the MdMADS12a in different types of tissues was monitored. The results demonstrated that MdMADS12 and MdMADS12a were co-expressed in the tissues tested, with MdMADS12 as the predominant form (Fig. 6b). Transcripts of both forms were detected in

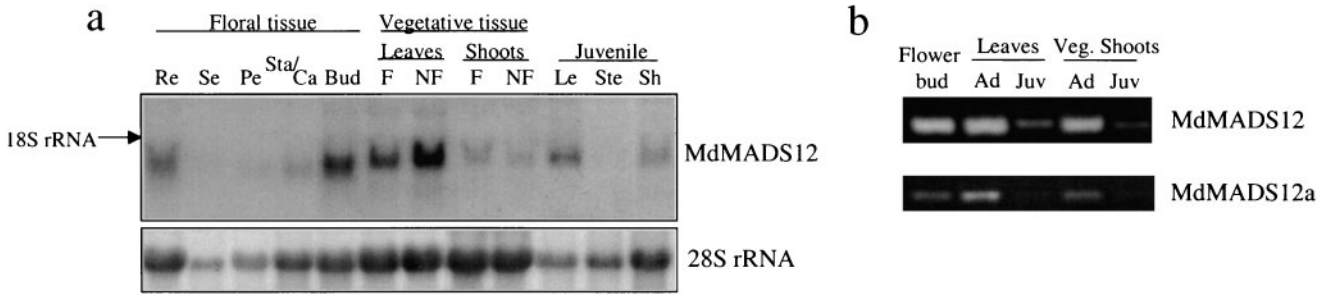

Fig. 6. Expression analysis of *MdMADS12* with (a) Northern blot and (b) RT-PCR. (a) Northern blot hybridization with MdMADS12 probe. Northern blot hybridizations of total RNA samples from several tissues of *Malus domestica*. Re, receptacle; Se, sepals; Pe, petals; St, stamens, Ca, carpels; all of mature flowers; Bud, flower bud; shoots and leaves of F, flowering trees (5-year-old); NF, non-flowering trees (5-year-old). Le, leaves; Ste, stem; Sh, shoot material from 2-year-old juvenile trees. (Upper panel) Labelled with specific radioactive cDNA probes of *MdMADS12* gene; position of 18S rRNA is indicated. (Lower panel) Labelled with radioactive 28S rRNA probe for correction of loaded amounts of RNA. (b) RT-PCR with total RNA samples of several apple tissues. Ethidium bromide-stained agarose gel of RT-PCR analysis of *MdMADS12* and *MdMADS12a*. Specific primers were used with total RNA from flower buds and vegetative shoots and leaves of 2-year-old seedlings (Juv) and 5-year-old adult (flowering) plants (Ad). Fragment length for *MdMADS12* RT-PCR is 230 bp, for *MdMADS12a* 200 bp.

leaves and shoots from both juvenile and adult trees, and in flower tissue. The MdMADS12/-12a expression ratios appeared not to be related to specific tissues or stages of development. In addition, the MdMADS12 and MdMADS12a transcripts were both found to contain at least the long 5′-non-translated region indicating that the use of the upstream transcription initiation site is not restricted to either MdMADS12 or MdMADS12a.

Northern blot analysis of MdMADS13, -14 and -15

Expression levels of MdMADS13, -14 and -15 mRNA were examined in several flower and non-flower apple tissues on Northern blots. The gene-specific cDNA probes consisted of the 3′-part of the coding region and the remaining non-coding sequence, to avoid cross-hybridization with other *MADS* box genes.

MdMADS13 mRNA was not detectable in vegetative tissues (Fig. 7a). In leaves and stem material no signal was detected on the Northern, even after long exposure times (not shown). Highest expression levels were observed in petals and stamens, in agreement with the suggestion that *MdMADS13* is a *B*-type gene. MdMADS13 mRNA was present in sepals and carpels, and in the receptacle, albeit at a low level.

Expression of MdMADS14 was detected primarily in carpels (Fig. 7b), which is in agreement with the suggestion that it is a *SHATTERPROOF* gene orthologue. MdMADS14 mRNA could not be detected in any vegetative tissues. In sepals a low level of MdMADS14 mRNA was present, and in stamens and receptacle a slightly higher level. Remarkably, it was not detected in petal tissue.

MdMADS15 expression was highest in stamens and in carpels (Fig. 7c), which is in line with a C-class function of this supposedly apple AGAMOUS orthologue. MdMADS15 mRNA was also clearly detectable in

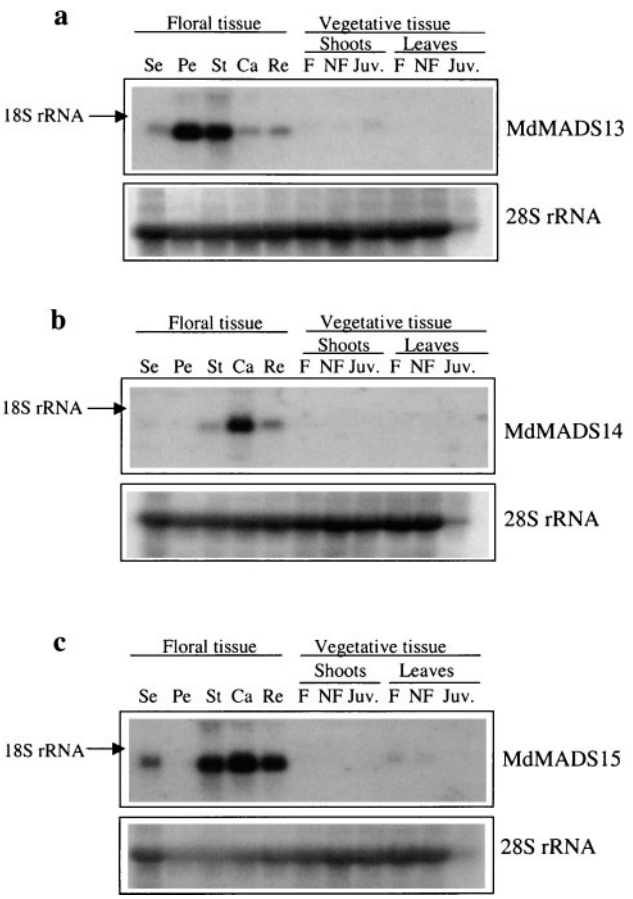

Fig. 7. Northern blot hybridizations with MdMADS13 (a), MdMADS14 (b) and MdMADS15 (c) probes. Northern blot hybridizations of total RNA samples from several tissues of *Malus domestica*. Se, sepals; Pe, petals; St, stamens; Ca, carpels; Re, receptacle; all of mature flowers; vegetative shoots and leaves of F, flowering trees (5-year-old), NF, non-flowering trees (5-year-old); Juv., juvenile trees (2-year-old). (Upper panels) Specific radioactive cDNA probes of *MdMADS13*, *-14* and *-15* genes; position of 18S rRNA is indicated. (Lower panels) 28S radioactive rRNA probe for correction of loaded amounts of RNA.

sepals and receptacles but absent from petals, and low levels of MdMADS15 expression were found in leaves of flowering and non-flowering 5-year-old trees.

RT-PCR analysis of MdMADS13, -14 and -15

The cDNA fragments of the MdMADS13, -14 and -15 were isolated from RNA extracted from vegetative shoots of 5-year-old apple trees. This implies that, although hardly or not detectable on Northern blots, expression of these genes is not limited to flower whorls. To determine whether MdMADS13, -14 and -15 mRNAs were expressed at earlier stages of development and in vegetative tissues, RT-PCR was performed with gene-specific primers on RNA from tissues of 2-year-old juvenile apple trees, and from pools of vegetative tissues of 5-year-old flowering and non-flowering trees.

A PCR product with the predicted length was detected with MdMADS13-specific primers in vegetative shoots of 5-year-old trees and of young, juvenile trees (2-year-old) (Fig. 8a). In leaves of 2-year-old seedlings, no signal could be detected, and in leaves of 5-year-old flowering and non-flowering trees the PCR product was hardly detectable after 35 PCR cycles. Control reactions for MdMADS13 as well as MdMADS14 and -15, containing as a template the starting total RNA without conversion to cDNA, did not produce a PCR product at all, indicating that the PCR fragments in Fig. 8 are derived from a cDNA template rather than from traces of genomic DNA.

Figure 8b shows that MdMADS14 was expressed in vegetative shoots of 5-year-old flowering and non-flowering trees, as well as in vegetative shoots of juvenile, 2-year-old seedlings. Furthermore, MdMADS14 was also present at low levels in leaves of 2-year-old trees, and more clearly visible in leaves of 5-year-old trees.

MdMADS15 mRNA was also observed in vegetative tissues with RT-PCR (Fig. 8c). Levels of expression of this particular mRNA may be related to age and developmental stage. In leaves and vegetative shoots of 2-year-old trees, the amount of PCR product appeared to be generally lower than those of 5-year-old vegetative shoots and leaves, in line with the faint signals observed on the Northern blots in leaf samples of 5-year-old trees, and the absence of detectable signals in RNA of juvenile leaves.

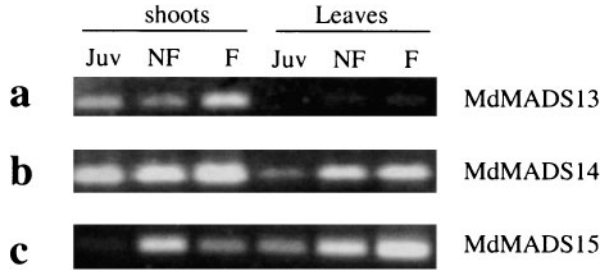

Fig. 8. RT-PCR with total RNA samples of several apple tissues. Ethidium bromide-stained agarose gel of RT-PCR analysis of *MdMADS13* (a), fragment length 830 bp, *MdMADS14* (b), fragment length 676 bp, and *MdMADS15* (c), fragment length 289 bp. Specific primers were used on cDNA synthesized with total RNA from vegetative shoots and leaves of 2-year-old seedlings (Juv), 5-year-old non-flowering plants (NF), and 5-year-old flowering plants (F).

Discussion

In this study a RT-PCR approach was used with degenerate MADS box primers on vegetative material with the intention of isolating *MADS* box genes possibly involved in the juvenile-to-adult phase transition. *MADS* box genes have been shown to play key roles in the initiation of flowering, and development of flower organs (Weigel, 1995; Theissen and Saedler, 1999), roots, leaves, seeds, and fruits (Buchner and Boutin, 1998; Gu *et al*., 1998; Liljegren *et al*., 1998; Perry *et al*., 1999; Alvarez-Buylla *et al*., 2000). For a woody plant like apple, insight in and possibly manipulation of the mechanism controlling the transition from vegetative to reproductive growth and the initiation of flowering is of great interest. Knowledge of genes involved in the onset of flowering may lead to experimental protocols that shorten the juvenile phase or identify genotypes with a relatively short vegetative period, which would be beneficiary to breeding programmes. Recently, *MADS* box genes were shown to be involved in the floral transition, both as inhibitors (*FLF*/*FLC* in *Arabidopsis*) (Sheldon *et al*., 1999; Michaels and Amasino, 1999) and as inducers (*PFG* in *Petunia*) (Immink *et al*., 1999). vegetative shoots were used as starting material to avoid the isolation of *MADS* box genes involved in flower formation. However, three out of four isolated sequences turned out to be homologous to flower whorl-determining genes, indicating that expression of the corresponding genes (*MDMADS13*, *-14* and *-15*) is not strictly confined to the reproductive tissues, and may be functional in other tissues and other developmental stages.

Characterization of MdMADS13

MdMADS13 falls within the *AP3* clade of *B*-type genes, and shows a high degree of similarity with *DEFICIENS* of *Antirrhinum* and *AP3* of *Arabidopsis*. Expression is highest in petals and stamens, confirming that this gene is an apple *AP3* orthologue.

Yao *et al*. have isolated and characterized the *PISTILLATA* homologue in apple, *MdPI*, and showed that expression of this gene resembles that of *PISTILLATA* in *Arabidopsis* (Yao *et al*., 2001). The genomic organization of the two genes is also similar. In addition, flowers of the naturally occurring apple mutant *Rae Ime*, which exhibits the same morphological changes as the *Arabidopsis PI* mutant, with sepals in place of petals, and an increased number of styles, was shown to have

a mutation in the *MdPI* gene. Interestingly, this apple mutant also gives rise to seedless fruit. The parthenocarpic behaviour may be due to the fact that fruit flesh is derived from sepal tissue. An impaired *B*-function results in enhanced sepal development in the first two whorls, which may lead to apple cortex development without pollination and fertilization (Yao *et al*., 2001).

In *Arabidopsis*, *PISTILLATA* and *APETALA3* are both required for specifying the *B*-type function, and disruption of either one results in similar phenotypes (Bowman *et al*., 1989; Hill and Lord, 1989). The *PI*/*AP3* dimer can bind to their *AP3* and *PI* gene promoters (Riechmann *et al*., 1996). In *Arabidopsis*, *B*-type function therefore relies on the presence of both *B*-type *MADS* box genes, although one or even two additional (floral tissue specific) MADS box proteins may be required for transcriptional activation of downstream genes (Honma *et al*., 2001). Similarly, *MdMADS13* (the apple *AP3* homologue) and *MdPI* may both be essential for the *B*-type function in apple flowers. Therefore, it is not unlikely that phenotypes similar to that of the *Rae Ime* mutant may be caused by mutations affecting the function of *MdMADS13*. Parthenocarpic apple varieties pose specific advantages for growers. Most importantly, fruit production without pollination eliminates the need for specific requirements (dependence on bees, pollinator varieties). Breeding programmes to produce commercial parthenocarpic varieties may benefit enormously from the availability of the sequence of both *MdPI* and *MdMADS13*. Transgenic approaches may be undertaken to down-regulate these genes. Alternatively, from the *MdPI* and *MdMADS13* sequences molecular markers can be developed for seedlessness, which may be used for selective breeding, which will speed up these breeding programmes considerably.

Characterization of MdMADS14 and MdMADS15

MdMADS15 expression in both the third and the fourth whorl agrees with a C-type function, and sequence homologies are highest with *AGAMOUS* and *AGAMOUS*-homologues. Several *AGAMOUS*-like genes from woody species have been identified (*DAL2* (*Picea abies*), Tandre *et al*., 1998; *SAG1* (*Picea mariana*), Rutledge *et al*., 1998; *LAG* (*Liquidambar styraciflua*), Liu *et al*., 1999; *CaMADS1* (*Corylus avellana*), Rigola *et al*., 1998; *GGM3* (*Gnetum Gnemon*), Winter *et al*., 1999, and *MdMADS10* from apple (Yao *et al*., 1999)). *MdMADS10* is likely to be the apple orthologue of *FBP7*/*FBP11*, specifying the '*D*-function' related to seed and fruit development (Colombo *et al*., 1997). Of the *Arabidopsis AGAMOUS*-like genes, *AGAMOUS* itself appears to be the only gene essential for development of the reproductive organs in *Arabidopsis*. The other AGL-like proteins act downstream of *AGAMOUS*, and may be under transcriptional control of this protein. The expression pattern of *MdMADS15*, with expression at a similar level in the third and fourth whorl, agrees with an *AGAMOUS*-like function in determining the identity of stamens and carpels, and it may therefore act earlier in development than *MdMADS14*.

The whorl-specific expression of MdMADS15 detected on Northern blots is partly comparable to the stamen- and carpel-specific expression of MASAKO C1, another *AGAMOUS* homologue within the Rosaceae family (Kitahara and Matsumoto, 2000). Interestingly, MdMADS15 is also expressed in sepals, which is unusual for an *AGAMOUS* homologue. This may be related to the fact that in apple, fruit development partly originates from sepal tissue, and it opens the possibility that MdMADS15 expression in sepals may be essential for the further development of sepal tissue into fruit flesh.

MdMADS14 expression was more restricted to the female flower organs than MdMADS15, It is most similar to *CaMADS1* (*Corylus avellana*) and *SHATTERPROOF1* and *-2* genes (formerly *AGL1*/*AGL5*). The latter genes are thought to be important for ovule development, and setting up the fruit dehiscence zone in *Arabidopsis* (Flanagan *et al*., 1996; Buchner and Boutin, 1998; Liljegren *et al*., 1998, 2000). SHP1 and SHP2 expression is carpel-specific. CaMADS1 of hazelnut is mainly expressed in carpel tissue, with low expression levels in male flower parts (Rigola *et al*., 1998). This is similar to the expression pattern of MdMADS14. Therefore, MdMADS14 may well be the apple orthologue of *SPH1* or *SPH2*, and as such play an important role in fruit and seed development.

Expression in vegetative tissue

The low mRNA levels in vegetative tissue of the floral organ determining *MdMADS* genes presented here may be background expression without any functional relevance. Alternatively, it may reflect expression at a considerable level in specialized cells that comprise only a small part of the analysed tissue.

It has been demonstrated by others that although expression levels are highest in flower whorls, some *MADS* box genes related to floral organ formation are also expressed at other stages of development and in other tissues. This is particularly true for *AGAMOUS*-like genes, which play a role not only in carpel development, but also at an earlier stage, at the onset of flowering (Hempel *et al*., 1997; Gu *et al*., 1998; Liljegren *et al*., 1998). *AGL8* is one of several genes (*AP1*, *LEAFY*, *CAULIFLOWER*) that promote flower formation in *Arabidopsis*, but it is also expressed in carpel tissue, and in vascular tissue of leaves, in which it was suggested to be a regulator for genes required for cellular differentiation (Hempel *et al*., 1997; Gu *et al*., 1998; Lijegren *et al*., 1998).

The observation that *MADS* box genes originally thought to be expressed exclusively in flowers, are also expressed and possibly functional in vegetative tissue of adult as well as in juvenile trees, has important implications for the biotechnological application of the promoters regulating the expression of these genes. Several groups are attempting to produce sterile plants, using *B*- and *C*-type gene promoters to target expression of cytotoxins to the reproductive parts of the flower. However, these results and those of others show that these promoters are not silent in vegetative tissue or at earlier stages of development. This implies that any cytotoxin under the transcriptional control of B-type and C-type promoters will be expressed in tissues other than the reproductive tissues, which may cause severe development and growth impairment in transgenic plants. This was found by the group of R Meilan, where transgenic experiments in *Populus trichocarpa* with cytotoxins (Barnase, DTA) under the control of a presumably flower-specific *MADS* box gene promoter caused severe growth defects (R Meilan, personal communication). Similar effects were observed with transgenic *petunia* containing the FBP7 promoter directing expression of Barnase (G Angenent, personal communication).

Characterization of MdMADS12

Two forms of MdMADS12 mRNA, differing at the 3′-end of the coding region, have been isolated. Specific amplification with genomic DNA indicated that these isoforms are transcribed from different loci, possibly as the result of gene duplication. As both mRNA isoforms are detected, both genes are active, with *MdMADS12* being the predominant form. Both isoforms can code for a protein, as in frame stopcodons are present not far from the point where the sequences diverge. Based on sequence similarity with known functional *MADS* box genes, MdMADS12 probably is the protein exerting the typical *MADS* box gene function. However, expression of *MdMADS12a* may interfere with this function. The MdMADS12 translated amino acid sequence contains both a glutamine-rich region (aa 185-195) and an acidic region (aa 241-245), similar to two regions in the *AP1* gene that were identified as being essential for trancriptional activation by *AP1* (Cho *et al*., 1999; Moon *et al*., 1999). The latter region is not present in *MdMADS12a*. It is tempting to speculate that the function of the *AP1* homologue *MdMADS12* may be (partly) inhibited through competition of *MdMADS12a*, which may have an impaired transcriptional activation domain. Expression changes in MdMADS12a would then affect MDMADS12 activity. The expression of both isoforms have been measured in vegetative and mature tissue. Expression of MdMADS12 and MdMADS12a appear to be correlated (Fig. 6b), which does not point to a regulatory function of MdMADS12a expression related to floral transition. However, additional, more detailed experiments are needed to rule out the possibility of such a regulatory mechanism.

MdMADS12 falls into the *AP1* clade of genes involved in floral transition and meristem development. It resembles two apple genes *MdMADS2* (Sung *et al*., 1999) and *MdMADS5* (Yao *et al*., 1999). *MdMADS2* has been studied in detail (Sung *et al*., 1999). Although a clear function for this gene could not be inferred from their results, it is suggested that *MdMADS2* is involved in the early development of the floral meristem and inflorescence. MdMADS2 as well as MdMADS5 expression was not detected in leaves, whereas MdMADS12 is expressed strongly in leaves, pointing to a clearly different function for *MdMADS12*. *MdMADS12* sequence resembles *AP1* and *AGL8* (*FRUITFULL*) of *Arabidopsis*, and the petunia *PFG* gene. From this, a role for *MdMADS12* in floral determination may be inferred. However, there are differences in MdMADS12 expression compared to these genes. For instance, MdMADS12 expression is completely absent from stems, whereas both AGL8 and PFG are expressed at high levels in this type of tissue (Mandel and Yanofsky, 1995; Immink *et al*., 1999).

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