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Molecular Cloning and Analysis of Two Flowering Related Genes from Apple (*Malus* × *domestica*)

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Abstract

Apple (*Malus* × *domestica* Borkh.) is the fourth fruit in importance and Iran ranks fifth in apple production in the world. Longevity of juvenility in apple extends breeding cycles and makes its breeding a tough job. To alleviate this barrier via genetic engineering, the genes involved in flowering and floral development of apple and their function must be identified and characterized. Most of these genes fall in a class of transcription factors named MADS-box genes. In the present research, we cloned and analysed the sequences and features of two of these genes, *MdMADS1* and *MdMADS3*, from apple 'Golden Delicious' for a deeper functional analysis in the near future. They were found to be homologs of SEP genes belonging to the class E genes involved in flower development and lied in the AGL2 clade of MADS-box genes in the phylogenetic tree made for apple and Arabidopsis MADS-box proteins. *In silico* studies exemplified that both genes had eight exons and seven introns with a long first intron of about 4 Kb and 3 Kb for *MdMADS1* and *MdMADS3,* respectively. The results showed that the structure of both genes has noticeably differed from other SEP-like genes in evolution.

Keywords: Flowering genes, Gene isolation, MADS-box genes, *Malus* × *domestica*, Phylogenetic tree.

Abbreviations: *AG, AGAMOUS; AGL, AGAMOUS-LIKE; AP1, APETALA1; bp, base pair; CAL, CAULIFLOWER; EST, expressed sequence tag; FBP, FLORAL BINDING PROTEIN; FLC, FLOWERING LOCUS C; FUL, FRUITFULL; Md-, Malus* × *domestica; PI, PISTILLATA; SEP, SEPALATA; SHP, SHATTERPROOF; STK, SEEDSTICK; SOC1, SUPRESSOR OF CONSTANS 1; TM5, Tomato MADS-box gene 5.*

INTRODUCTION

Apple (*Malus* × *domestica* Borkh.) is the fourth fruit in importance after citrus, bananas and grapes and Iran with a production of 2.43 million metric tons ranks fifth after China, the United States, Turkey and Poland (FAO, 2012). Improvement of this plant is a difficult job due to the longevity of its juvenility which extends breeding cycles up to decades. To alleviate this major breeding barrier, genetic manipulation of the juvenility can be a prominent alternative to classic methods; however, it requires the genes controlling the transition from vegetative phase to reproductive phase to be identified and functionally characterized. Most of these genes and also the majority of the genes active in the determination of meristem identity and the development of floral organs lie in a class of transcription factors named MADS-box genes. Although a few studies have demonstrated that the MADS domain of MADS-box proteins in fruit trees plays a similar role as in grasses (Sundstrom and Engstrom, 2002), there is little information about the features of this domain in trees (Sung and An, 1997). Moreover, SEP genes functioning as members of the class E genes in the ABCDE model of flower development are among MADS-box genes. It has been demonstrated that SEP genes have expression in all floral whorls (Sun and An, 1997; Sung *et al*., 2000; Ditta *et al*., 2004). The overexpression of one of SEP homologs in apple, *MdMADS1*, has not caused any change in the flowering time of tobacco (Sung and An, 1997); nevertheless, it seems that the function of the gene can be further investigated through antisense or co-suppression methods.

In the present research, our aim was to clone and characterize the sequences and the features of two homologs of SEP genes from the 'Golden Delicious' cultivar of apple namely, *MdMADS1* and *MdMADS3* to further analyse their function via gene knockdown approaches.

MATERIALS AND METHODS

Plant tissues needed for RNA extraction were collected from the leaves of five–year old apple 'Golden Delicious' trees and frozen using liquid nitrogen. RNA extraction was performed from buds and leaves using LiCl method. The quantity and quality of extracted RNA was verified using spectrophotometer and agarose gel electrophoresis. All solutions were DEPC-treated. Suitable amount of RNA used in RT reaction by means of the enzyme SuperScript II® (Invitrogen) using manufacturer's protocols. The forward and reverse primers employed to clone these genes are listed in table 1. From RT reaction, 1 µl was added to the PCR mixture (Table 2) to amplify the fragments. The thermal program for PCR reaction was as table 3.

To clone the RT-PCR fragments, pGEM-T Easy kit (Promega) was exploited using manufacturer's protocols. Sequencing was carried out through sequencing facilities of VIB-UGent (Belgium). Sequence analysis and alignments were performed via Mega 5 (Tamura *et al*., 2011). The sequences were downloaded from diverse databases and used for constructing a phylogenetic tree (Table 4) using neighbour-joining algorithm (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

In this study, we provide the report of two MADS-box genes, *MdMADS1* and *MdMADS3*, which were isolated from Malus domestica based on RT-PCR. The genes found to be homologous to Arabidopsis *SEP*-like genes. The isolated sequences for *MdMADS1* and *MdMADS3* were cloned in pGEM-T Easy vector and the resulted vectors designated pNM101 and pNM102, respectively. The RT-PCR and colony-PCR results of the isolated genes have been demonstrated in Fig.1.

MdMADS1 gene was first isolated and cloned by Sung and An (1997) from apple cv. Fuji. The cDNA of this gene being 992 bp has an ORF which is 738 bp, a 5'-UTR of 29 bp long and a 3'-UTR with a length of 222 bp. In the present study, the coding region of the gene with a length of 741 bp was isolated and cloned. This region encodes a 246 amino acid long polypeptide which belongs to MADS-box family. The gene *MdMADS1* isolated in this research had a 99.9% similarity to the original *MdMADS1* with the only difference in the nucleotide position 218 being G in *Md-*

MADS1 and T in *MdMADS1* from 'Golden Delicious' which makes a shift in amino acid 63 replacing Methionine with Isoleucine. This little difference makes the isolated *MdMADS1* gene identical to *MdMADS8* instead of the original *MdAMDS1*. Another possible explanation can be the error of *Taq* DNA polymerase in RT-PCR reaction which might have replaced G with T in the isolated *MdMADS1*. This sequence was isolated using RNA extracted from the buds, while it has been reported that this gene expresses in the floral organs (Sung and An, 1997). The amino acid sequence of this protein had a similarity of 93% with *MdMADS9*, 79% with *SEP1/AGL2* and 74% with *SEP2/AGL4* (Sung *et al*., 1999). Therefore, it lied in the AGL2 clade of MADS-box proteins (Fig. 2). Furthermore, the expression pattern of *MdMADS1* is comparable to that of *AGL2*. During first and middle stages of flower development, *AGL2* shows a high expression in all floral whorls. While the flower development continues, *AGL2* expression undergoes a dramatic decrease. The expression of *AGL2* in ovules, embryos and seed coat has been also high (Flanagan and Ma, 1994). *AGL2* expression has been recognized in the leaves (Ma *et al*., 1991); however, *MdMADS1* transcripts have not been detected in the vegetative organs (Sung and An, 1997).

MdMADS3 was first isolated from 'Fuji' apple (Sung *et al*., 2000), the cDNA of which has a length of 1104 bp and contains an ORF encoding a protein with 248 amino acids, a 5'-UTR of 81 bp long and a 3'-UTR of 276 bp long. It was isolated in this research from 'Golden Delicious' being 98.9% similar to *MdMADS3* from 'Fuji'. The 5'-UTR of the former had 6 times less repetition of GA than that of the latter. Perhaps, this type of dissimilarity can be used in distinguishing of the two cultivars through SSR marker development. The amino acid sequence of this gene was as 99% similar as that of *MdMADS7* (Yao *et al*., 1999). In the amino acid 181, there is substitution of histidine in *MdMADS7* for glycine in *MdMADS3*. Further analysis revealed that *MdMADS3* had 83% similarity with MdMADS6. Upon drawing phylogenetic tree, *MdMADS3* stood along with *AGL2* (Fig. 2). In comparison with Arabidopsis genes, it showed the highest similarities with *SEP1/AGL2* (63%), *SEP2/AGL4* (61%) and *SEP3/AGL9* (59%).

Plant MADS box genes were realized to be transcription factors that regulate floral organ identity; nevertheless, several reports have since been issued about their regulation of other developmental processes, such as flowering time, fruit ripening, root growth, dehiscence, ovule and female gametophyte development and the determination of meristem identity of vegetative, inflorescence, and floral meristems (Zhang and Forde, 1998; Ng and Yanofsky, 2001; Giovannoni, 2004; Whipple *et al*., 2004; L. Colombo *et al*., 2008; Liu *et al*., 2009). Researches on model species such as Antirrhinum majus, Arabidopsis thaliana, Petunia hybrida, Oryza sativa, and Zea mays have been shown that these functions are mostly conserved among angiosperms (Schwarz-Sommer *et al*., 2003; Vandenbussche *et al*., 2003; Kater *et al*., 2006).

Genetic researches have also exemplified that closely related family members MADS-box genes have redundant or overlapping functions. For example, the closely related SEPALLATA (SEP) genes, *SEP1, SEP2*, and *SEP3*, act redundantly to specify petal, stamen, and carpel identity. Single sep mutants have no phenotype, and a sep1 *sep2 sep3* triple mutant was required to establish the developmental role of these genes (Pelaz *et al*., 2000). The closely related SHATTERPROOF1 (SHP1) and SHP2 genes act redundantly to regulate the formation of the silique dehiscence zone (Liljegren *et al*., 2000). Furthermore, *SHP1* and *SHP2* act redundantly with *AGAMOUS* (AG), which specifies stamen and carpel identity, and SEEDSTICK (STK), which controls the development of the funiculus, to specify ovule identity (Pinyopich *et al*., 2003). Moreover, several phylogenetic studies of the MADS domain family have revealed that related genes within a phylogenetic clade tend to share similar expression patterns (Purugganan *et al*., 1995; TheiBen *et al*., 1996; Riechmann and Meyerowitz, 1997; Alvarez-Buylla *et al*., 2000b). These findings suggest that redundant functions might be a noticeable characteristic of closely related members in the MADS-box gene family.

SEP-like genes are generally encoded by multigene families and it has been supposed that

the gene duplications occurred along angiosperm evolution, and that the number of SEP-like genes was diverse in different species (Theissen *et al*., 2000; Becker *et al*., 2000; Zahn *et al*., 2005). Four genes in the A. thaliana genome (Ma *et al*., 1991; TheiBen; 2001 and Ditta *et al*., 2004), six in both petunia (Zahn *et al*., 2005) and wheat (Paolacci *et al*., 2007), five in both rice (Nam *et al*., 2004) and oil palm (Adam *et al*., 2006), three in Asparagus sp. (Kanno *et al*., 2006), two in peach (Tani *et al*., 2009) and one in Alpinia hainanensis (Song *et al*., 2010), Zostera japonica (Kakinuma *et al*., 2011) and Fragaria ananassa (Seymour *et al*., 2011) are derived from cDNA or gDNA sequences. Genome-wide analysis indicates that the *M. domestica* genome contains several MADS-box genes including at least seven SEP-like genes (Fig. 2), suggesting that the process of floral specification in M. domestica could be similar to its relatives.

Based on in silico studies performed in this research, the genomic locus of *MdMADS1* was 5748 bp long at about 5 Mb from the beginning of the chromosome 17 and that of *MdMADS3* was 5260 bp at about 27 Mb from the beginning of the chromosome 14 of apple. Both genes had eight exons and seven introns with a long first intron of about 4 Kb and 3 Kb for *MdMADS1* and *MdMADS3*, respectively. The exon–intron structure of MIKC-type MADS-box genes is well conserved (Henschel *et al*., 2002; Tanabe *et al*., 2005). In the plants studied, the ORF of the MIKC-type MADS-box genes is interrupted by six (e.g., A. thaliana SEP1 and SEP2 in SEP1/2 subclade) to seven (e.g., SEP3 and *SEP4* in *AGL2* clade, *AGL6* and *AGL13* in *AGL6* clade, and AP1, CAL, and FUL in FUL clade) introns. In the former type, lengths of ORF coding region in Es1–7 are 185, 82, 62, 100, 84, 146–149, and 94–127 bp, respectively, and those in Es1–8 in the later type are 182–185, 73–85, 62–71, 100, 42, 36–42, 128–173, and 34–115 bp, respectively (Johansen *et al*., 2002). The exon–intron structure of *MdMADS1* and *MdMADS3* genes is more similar to those of the *SEP3* and SEP4 than those of the *SEP1* and SEP2 in the *AGL2* clade; while in terms of amino acid sequence, *MdMADS1* protein was more similar to *SEP1/2* subclade and *MdMADS3* found to be a little different from *SEP1, SEP2, SEP3* and *SEP4* (Fig. 2). These results exemplify that the structure of both genes has noticeably differed from other SEP-like genes in evolution.

According to the ABCDE model proposed to explain floral organ formation, the individual and combined activities of five classes (A, B, C, D, and E) of homeotic genes (Theiβen, 2001, Becker and Theiβen, 2003, Ditta *et al*., 2004, Adam *et al*., 2007a, Adam *et al*., 2007b and Liu *et al*., 2010). In A. thaliana, A-function is provided by the AP1 and AP2 genes, B-function by the *AP3* and *PI* genes, C-function by the AG gene, D-function by the STK gene, and E-function by the *SEP* genes (i.e., *SEP1, SEP2, SEP3*, and *SEP4*). The four *SEP* genes in A. thaliana, *SEP1–SEP4*, are well characterized, all of which play important roles for specifying the identity of all four whorls of the floral organ and for floral meristem identity (Ditta *et al*., 2004), and the similar functions of SEP-like genes have been shown not only in other dicot species (Vandenbussche *et al*., 2003b and Rijpkema *et al*., 2009) but also in monocot species (Adam *et al*., 2007b, Paolacci *et al*., 2007 and Song *et al*., 2010). In the case of M. domestica, the expression of the *MdMADS1* gene, which is a member of *SEP1/2* subclade (Fig. 2), was in all floral organs and young fruits but not in leaves. The expression was higher at the early stages of flower and fruit development, suggesting that *MdMADSl* plays a major role in the initiation of reproductive organ developments. The *MdMADS1* gene expression pattern is similar to that of *AGL2* of Arabidopsis. 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 the *MdMADS1* transcript was not found in the vegetative organ. Unfortunately, the exact role of *AGL2* genes are not elucidated yet, but, the studies on their homologs in petunia and tomato have revealed a role for these genes as medi-

ators between floral meristem identity genes and floral organ identity genes (Angenent *et al*., 1992; Pnueli *et al*., 1994).

Taken together, it appears that *MdMADS1* is a member of the *AGL2* subfamily. Unfortunately, the function of the *AGL2* subfamily is largely unknown, transgenic phenotypes of FBP2 and TM5 deficient plants suggest a role in mediating between meristem and organ identity genes (Angenent *et al*., 1994; Pnueli *et al*. 1991). Ectopically expressed *MdMADS1* gene under the control of 35S promoter in tobacco did not cause any alteration of flower or seed development (Sung and An, 1997). In order to reveal the functional role of *MdMADS1*, it may be necessary to employ either antisense or co-suppression approaches in the homologous apple plant to achieve reduction of the gene expression. The expression of *MdMADS3* was first detected at the stage 3 in three internal whorls of floral primordia; nevertheless, no expression was observed in the younger floral primordia and/or in the inflorescence meristem (Sung *et al*., 1999).The expression pattern of *MdMADS3* is similar to those of FBP2 from petunia, TM5 from tomato, and *AGL9* from Arabidopsis, which are expressed in petals, carpels, and stamens (Angenent *et al*., 1992; Pnueli *et al*., 1994; Mandel and Yanofsky, 1998). The genes FBP2, TM5, and *AGL9* are expressed after the onset of the meristem-identity genes, but before the activation of organ-identity genes, suggesting a possible role as mediators between the floral meristem and floral organ-identity genes. The egm1 and egm3 genes from eucalypt and the DEFH49 gene from Antirrhinum majus are also expressed in the inner three whorls of the flower (Davies *et al*., 1996; Southerton *et al*., 1998).

With complementation of genome sequencing and expressed sequence tag (EST) assembling in some model plants such as Arabidopsis, rice, and wheat, 107, 73, and 45 MADS-box genes have been annotated, respectively (Kofuji *et al*., 2003, Pařenicová *et al*., 2003, Nam *et al*., 2004, Zhao *et al*., 2006a and Paolacci *et al*., 2007), demonstrating that the MADS-box genes in plants form a large family that plays distinct roles in flower development and organ differentiation, and that fully understanding the molecular mechanisms for M. domestica reproductive organ formation, in which MADS-box genes may be involved, will be needed. Recently, EST databases for apple have been provided and its genome has been fully sequenced (Velasco *et al*., 2010). Further experiments for isolation and functionally characterization of apple MADS-box genes related to its flowering time and reproductive organs development are currently being carried out.

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Tables

Table 1. Forward and reverse primers used for cloning MADS-box genes in RT-PCR reaction.

Gene	Forward primer	Reverse primer
MdMADS1	GGCGGATCCATGGGGAGAGGAAGAGTG	CGCATGGATCCTCAAAGCATCCATCCAG
MdMADS3	GCAGGATCCCAGTTTGTCTACCTCTGA	CGCGGATCCGTATATACAAATTGGTCTC

Table 2. Polymerase chain reaction mixture for amplification of the genes from RT reaction.

Ingredients	Amount
PCR water	38.5 µl
Forward primer of the gene of interest (20 uM)	1 µl
Reverse primer of the gene of interest (20 uM)	1 µl
Actin-5P primer (20 uM)	1 µl
Actin-3P primer (20 uM)	1 µl
10 mM dNTPs	1 µl
10x Platinum buffer	5 µl
Platinum Taq DNA Polymerase	0.5 µl

Table 3. Thermal programming of the PCR reaction.

Temperature	Time duration
94 °C	2 min
32 cycles:	
94 °C	30 sec
Taa (50/55/60°C)	30 sec
72 °C	30 sec per 0.5 kb
Final extension:	
72 °C	10 min

Table 4. The genes (proteins) used in this research in making phylogenetic tree and their gi-numbers in the public databases GeneBank/EMBL/DDJB.

Malus × *domestica*		*Arabidopsis thaliana*	
Gene name	**GI number**	**Gene name**	**GI number**
MdMADS3	5777904	SEP1/AGL2	52548008
MdMADS1	3290209	SEP2/AGL4	52548054
MdMADS2	3947985	SEP3/AGL9	334182820
MdMADS4	5777906	SEP4/AGL3	330250646
MdMADS5	110681903	AP1/AGL7	332196766
MdMADS6	351602211	AP3	332645695
MdMADS7	302398909	AG	3915597
MdMADS8	3646334	FLC	332004118
MdMADS9	3646336	PI	332005434
MdMADS10	3646326	AGL8/FUL	1004365
MdMADS11	3646340	AGL1/SHP1	113511
MdMADS11.1	302398915	AGL5/SHP2	113515
MdMADS12	32452882	AGL6	330255488
MdMADS13	16973294	CAL/AGL10	259016368
MdMADS14.1	302398885	AGL11/STK	12229648
MdMADS14	16973296	AGL12	332197095
MdMADS15	16973298	AGL13	332646637
MdMADS16	189339107	AGL14	332657662
MdMADS16.1	302398887	AGL15	332004558
MdMADS17	302398889	AGL16	332646109
MdMADS18	302398891	AGL17	330252237
MdMADS19	302398893	AGL18	332646129
MdMADS20	302398897	AGL19	332659284
MdMADS21	302398899	SOC1/AGL20	17433202
MdJOINTLESS	122056647	AGL24	332659522
MdPI	12666535	AGL71	332008757
MdAGL	33308109	AGL72	32402406
MdSOC1a	114386386	AGL79	32402440
MdSOC1ak	268327050		
MdSOC1c	295684203		

Figures

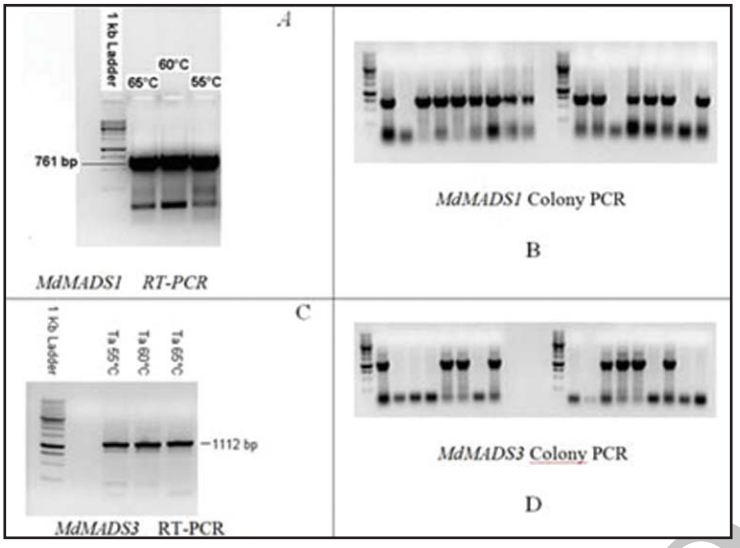

Fig. 1. Agarose (1%) gel electrophoresis of RT-PCR and colony-PCR of the isolated genes. A. RT-PCR of *MdMADS1-Golden*, B. Colony-PCR of *MdMADS1-Golden*, C. RT-PCR of *MdMADS3-Golden*, D. Colony-PCR of *MdMADS3-Golden.*

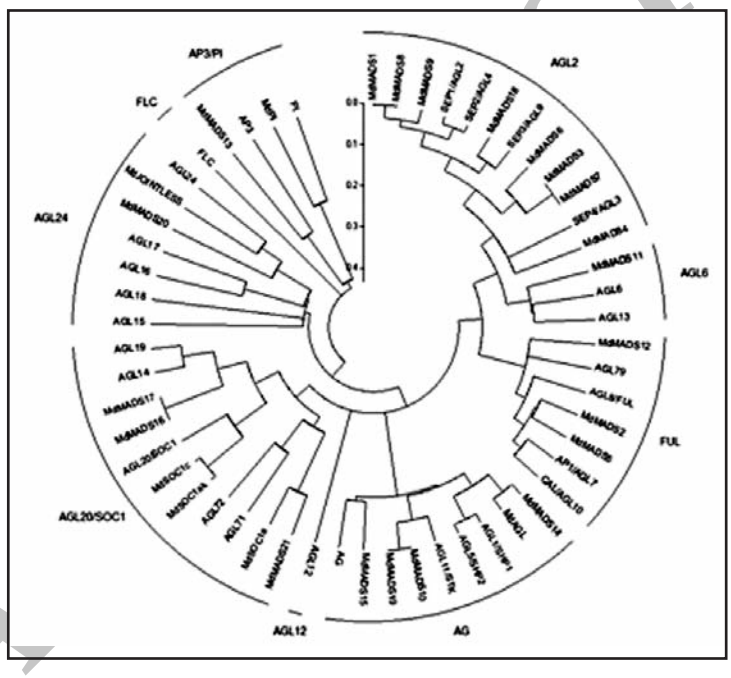

Fig. 2. Evolutionary relationships of apple and *Arabidopsis* MADS-box genes. AGL2 clade of MADS-box genes comprises Md-MADS1 and MdMADS3 genes isolated in this research. The genes with "Md-" prefix are from apple and the others are from Arabidopsis. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 10.01153205 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 55 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 122 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).