Genome-wide Identification and Analysis of the MADS-box Gene Family in Melon

Xin Hao, Yu Fu, Wei Zhao, Lifei Liu, Rengui Bade, Agula Hasi, and Jinfeng Hao¹

Inner Mongolia Key Laboratory of Herbage and Endemic Crop Biotechnology, College of Life Sciences, Inner Mongolia University, Hohhot, Inner Mongolia, People's Republic of China

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ABSTRACT. The MADS-box gene family encodes a type of transcription factor, and plays a key role in the growth and development of plants. Here, we identified 62 MADS-box genes in the melon (*Cucumis melo*) genome using bioinformatics methods. These genes were divided into type I M α , M γ , and M δ subfamilies (26 members) and type II MIKC^c subfamilies (36 members) by phylogenetic analysis. There were no genes in type II AGL12, BS, TM8, and MIKC^{*} subfamilies, and type I M β subfamilies. Conserved motif analysis showed that all motifs had a subfamily-specific distribution except the M domain. The expression analysis of the MADS-box genes showed different expression characteristics. In summary, this study is the first to identify melon MADS-box genes and analyze their gene structures, subfamily distribution, and expression characteristics. These results provide a foundation for investigating the functions of the melon MADS-box genes.

The MADS-box gene family, which encodes a type of transcription factor, is a regulatory gene family found widely in eukaryotic genomes. Researchers successively found MADS-box genes in fungi (Passmore et al., 1988), animals (Norman et al., 1988), and plants (Sommer et al., 1990; Yanofsky et al., 1990). The MADS-box genes received their name from the first initials of four transcription factors, MCM1 (*Mini Chromosome Maintenance 1*) from *Saccharomyces cerevisiae* (Passmore et al., 1988), AG (AGAMOUS) from *Arabidopsis thaliana* (Yanofsky et al., 1990), DEF (Deficiens) from *Antirrhinum majus* (Sommer et al., 1990), and Serum Response Factor (SRF) from *Homo sapiens* (Norman et al., 1988). The proteins contain a conserved region of ≈ 58 –60 amino acids in their N-termini called the MADS-box (Becker et al., 2000; Theissen et al., 2000), which is a domain-binding DNA sequence that recognizes CARG boxes and regulates the expression of target genes (De Bodt et al., 2003). Studies have shown that plant MADS-box transcription factors play an important role in the control of floral organ and fruit development, flowering time, and gametophyte cell division (Ditta et al., 2004; Fang and Fernandez, 2002; Favaro et al., 2003; Liljegren et al., 2000; Ma, 2005; Michaels et al., 2003; Pinyopich et al., 2003).

Plant MADS-box genes can be divided into two categories, types I and II (Alvarez-Buylla et al., 2000). In comparison with type II, type I genes have a simpler gene structure and lack the K domain. In *Arabidopsis*, type I MADS-box genes contain M α , M β , M γ , and M δ subfamilies (Pařenicová et al., 2003). Studies on type II genes have been relatively extensive, but type I genes have been less researched and are less well understood. Type II MADS-box genes can also be further subdivided into MIKC^{*} and 13 MIKC^c subfamilies (Díaz-Riquelme et al., 2009; Henschel et al., 2002): AG, AGL6, AGL12, AGL15, AGL17, API/FUL, AP3/PI, FLC, SCO1, SEP, SVP, BS, and TM8. In

addition to the M domain, MADS-box genes have three other conserved domains: the K domain, which is relatively conserved and characterized by a coiled-coil structure, only exists in type II genes and is the basis of the distinction between type I and II genes (Davies et al., 1996; Ma et al., 1991; Pnueli et al., 1991); the I domain, which is located between the M and K domains, may contribute to specificity in the formation of DNA-binding dimers (Riechmann et al., 1996); and the C domain, which has multiple functions, can activate transcription in some MADS-box genes of yeast cells (Kramer et al., 1998). Compared with MIKC^c-type genes, MIKC^{*}-type genes contain a longer I domain and a shorter K domain.

MADS-box genes play a significant role in plant development processes, especially in floral organ development (Causier et al., 2002). The ABC model of floral development was established via studies on model plants, such as *Arabidopsis* and *A. majus* (Coen and Meyerowitz, 1991). Subsequently, many MADS-box genes and proteins were fully researched and explored, and the widely accepted ABCDE model was proposed (Honma and Goto, 2001). In this model, A-function genes determine sepal development, A- and B-function genes codetermine petal development, B- and C-function genes codetermine stamen development, C-function genes control carpel development, D-function genes determine ovule development, and E-function genes regulate the development of various organs (Kaufmann et al., 2005; Ma, 2000; Pelaz et al., 2000).

Melon, a diploid eudicot plant, is an annual herbaceous vine. Widely cultivated, melon is an important horticultural crop and one of the most popular fruit. Melon has considerable economic value, especially in the Mediterranean and central Asian countries. Through plant genome projects, genome-wide sequencing has been completed for many model plants, such as corn [*Zea mays* (Schnable et al., 2009)], sorghum [*Sorghum bicolor* (Paterson et al., 2009)], soybean [*Glycine max* (Schmutz et al., 2010)], and grape [*Vitis vinifera* (Jaillon et al., 2007)]. The genome sequence and gene annotations for melon have also been published (Garcia-Mas et al., 2012), which has established a foundation for studying the melon MADS-box gene family. There has been relatively extensive

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¹Corresponding author. E-mail: haojindd@163.com.

research on MADS-box genes in arabidopsis (Pařenicov et al., 2003), poplar [*Populus trichocarpa* (Leseberg et al., 2006)], rice [*Oryza sativa* (Arora et al., 2007)], grape (Daz-Riquelme et al., 2009), soybean (Shu et al., 2013), and cucumber [*Cucumis sativus* (Hu et al., 2012)], but, so far, in-depth research on melon MADS-box genes has not been performed. Melon, as a model plant for fresh fruit, has important scientific value and significance warranting further discussion and research on the melon MADS-box gene family.

Materials and Methods

IDENTIFICATION OF MADS-BOX GENES IN MELON. The melon genome was downloaded from the MELONOMICS database (Pere and Jordi, 2012). First, a protein database was established with the genome-wide amino acid sequence using the BioEdit 7.2.0 software (Hall, 1999), and then a Hidden Markov Model (HMM) for the SRF-TF (PF00319) and K-box (PF01486) was found from the Pfam database (Finn, 2014) and used as a probe to perform local protein alignment with the local Basic Local Alignment Search Tool (BLASTp) program. When conducting the alignment, the program was set to the default parameters and the cutoff E-value was 0.001. The alignment results were used to identify candidate genes, and then conserved domain analysis was conducted with the SMART program (Letunic et al., 2015; Schultz et al., 1998) to remove repetitive and redundant sequences. Finally, the complete set of melon MADS-box family genes was obtained.

INTRON-EXON STRUCTURE ANALYSIS AND CHROMOSOMAL LOCATIONS. In the melon database, the CDS and DNA sequences of 62 melon MADS-box genes were numbered using the FASTA format. The distribution of exons and introns in the melon MADS-box genes was analyzed using the online software GSDS (Hu et al., 2015). According to the positional information for the melon MADS-box genes, we used the MapDraw software (Liu and Meng, 2003) to map the distribution of the melon MADS-box genes on the chromosomes.

SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS. The arabidopsis MADS-box genome was downloaded from the *Arabidopsis* Information Resource (TAIR) website (Rhee et al., 2003). The cucumber MADS-box genome was downloaded from the cucumber database (BGI, 2009), which was released by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (IVF-CAAS). Multiple sequence alignment was performed using CLC Sequence Version 7.6.1 (Qiagen, Duesseldorf, Germany) with default parameters. A phylogenetic tree was built with the MADS-box genes of arabidopsis, cucumber, and melon using the neighbor-joining method in the MEGA 4.1 software (Tamura et al., 2007), with the following parameters: Poisson correction, pairwise deletion, and bootstrapping (1000 replicates). All of the melon MADS-box genes were classified into subfamilies based on the phylogenetic tree.

ANALYSIS OF CONSERVED MOTIFS. MEME version 4.10.2 (Bailey and Elkan, 1994), a software tool for predicting conserved sequences of genes, was used to analyze the conserved motifs of the melon MADS-box genes (maximum number of motifs: 10, motif width >6 and <200).

EXPRESSION ANALYSIS WITH REVERSE-TRANSCRIPTION POLYMERASE CHAIN REACTION AND QUANTITATIVE RT-PCR. Plants of melon cultivar Hetao were grown under experimental field conditions from May to July 2014 at Inner Mongolia University, Hohhot, China. At the flowering stage, we collected

hermaphrodite flowers and fruit at 0 d after pollination (DAP), as well as young roots, leaf blades, and tender stems. Meanwhile, self-pollination was performed by hand and the pollination time was accurately controlled. Only one fruit was kept for each plant. The mesocarp of fruit (15 and 35 DAP) was collected. All harvested samples were immediately washed with sterile water, frozen in liquid nitrogen, and stored at -80°C for RNA extraction. TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to extract RNA from all melon samples, and electrophoresis test results showed clear RNA bands and good integrity. Reverse transcription was performed with PrimeScriptTM RT Master Mix (TaKaRa, Dalian, China) in a 10- μL reaction system containing 2 μL 5 $\times$ buffer, 500 ng RNA, and RNase-free dH₂O up to 10 μL . The reaction conditions were 37 $^{\circ}\text{C}$ for 15 min and 85 $^{\circ}\text{C}$ for 5 s. Aliquots of 4 and 2 μL were taken as templates for reverse-transcription polymerase chain reaction (RT-PCR) and quantitative TR-PCR (qRT-PCR), respectively, after the reverse transcription product was diluted 10 times. We designed primers for the experiment with the Primer 5.0 software (Premier Biosoft, Palo Alto, CA); during the design process, we tried to avoid the conserved regions of MADS-box genes and design primers across long introns as far as possible to remove potential DNA contamination. After primer design was completed, the primers were aligned to the melon database to confirm their specificity. All PCR products were run on 2% agarose gels. For qRT-PCR, a 25- μL reaction system was used containing 12.5 μL 2 $\times$ SYBR Premix Ex Taq II (TaKaRa), forward and reverse primers diluted to 0.4 μM , and the templates mentioned above. The reaction conditions were 95 $^{\circ}\text{C}$ for 30 s, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 5 s and 60 $^{\circ}\text{C}$ for 30 s. The reactions were performed with a Chromo 4 Real-Time PCR Detector (Bio-Rad, Hercules, CA). The relative expression of genes was calculated by the $2^{-(\Delta\Delta\text{Ct})}$ method. At least three technical replicates were performed for each reaction and three biological samples were used for each gene.

Results

IDENTIFICATION, GENOMIC DISTRIBUTION, AND ANNOTATION OF MADS-BOX GENES IN MELON. To identify melon MADS-box family genes, we used the HMM model for the SRF-TF (PF00319) and K-box (PF01486) to conduct local protein alignment and collected the results as candidate proteins. After we removed duplicated and functionally redundant genes, 62 MADS-box genes remained. Analysis with the SMART software shows that all 62 genes had a typical MADS-box domain. We named the genes *CmMADS01–CmMADS62* according to their distribution of subfamily and collected relative statistics about them (Table 1). Table 1 shows the corresponding statistics and analysis, including amino acid length, number of exons and introns, chromosomal assignment, and subfamily classification.

We mapped the chromosomal locations of the genes based on their location information using the MapDraw software (Fig. 1). This showed that the gene family was differentially distributed across the chromosomes. Chr8 and Chr11 both had eight genes, whereas Chr5 had no genes. The remaining chromosomes contained one to seven genes. Chr1 had two type II genes but no type I genes, whereas Chr2 and Chr10 only had one type I gene each but no type II genes. The gene distribution on the other chromosomes was about equal.

Table 1. Identity of 62 MADS-box genes from a local blast against melon genome and removed repetitive and redundant sequences with the SMART program (Letunic et al., 2015). Obtained the information including amino acid length, number of exons and introns, and chromosomal assignment. The subfamilies were classified based on the phylogenetic tree.

Gene	Gene identification	Location	Chromosome no.	Protein length (no. of amino acids)	Exons (no.)	Introns (no.)	Subfamily
CmMADS01	MELO3C002691T1	Scaffold00001 5563523–5568056	12	256	8	7	AG
CmMADS02	MELO3C007181T1	Scaffold00007 1322082–1329014	8	237	9	8	AG
CmMADS03	MELO3C007181T2	Scaffold00007 1322082–1329014	8	242	9	8	AG
CmMADS04	MELO3C007181T3	Scaffold00007 1322082–1329266	8	262	9	8	AG
CmMADS05	MELO3C022209T1	Scaffold00051 2262147–2270592	9	230	9	8	AG
CmMADS06	MELO3C022516T1	Scaffold00052 2287507–2292344	11	187	7	6	AGL6
CmMADS07	MELO3C022516T2	Scaffold00052 2287507–2292344	11	249	8	7	AGL6
CmMADS08	MELO3C024001T1	Scaffold00064 990832–994894	4	251	8	7	AGL15
CmMADS09	MELO3C019192T1	Scaffold00036 4133578–4136082	N/A ^z	135	4	3	AGL15
CmMADS10	MELO3C003502T1	Scaffold01596 1775790–1799198	4	238	9	8	AGL17
CmMADS11	MELO3C003502T2	Scaffold01596 1775876–1799198	4	239	9	8	AGL17
CmMADS12	MELO3C007700T1	Scaffold00007 4765228–4767238	8	150	4	3	AGL17
CmMADS13	MELO3C018049T1	Scaffold00031 3970196–3980905	7	190	6	5	AGL17
CmMADS14	MELO3C002050T1	Scaffold00001 900368–905507	12	248	8	7	AP1/FUL
CmMADS15	MELO3C011409T1	Scaffold00014 5441565–5446683	3	151	3	2	AP1/FUL
CmMADS16	MELO3C003778T1	Scaffold01596 4117765–4122123	4	270	7	6	AP3/PI
CmMADS17	MELO3C010515T1	Scaffold00013 3450090–3454750	7	248	7	6	AP3/PI
CmMADS18	MELO3C022315T1	Scaffold00052 754375–771989	11	189	5	4	FLC
CmMADS19	MELO3C005617T1	Scaffold00005 6499878–6505422	9	151	6	5	SCO1
CmMADS20	MELO3C005617T2	Scaffold00005 6499878–6505422	9	223	7	6	SCO1
CmMADS21	MELO3C005393T1	Scaffold00005 4706570–4715038	9	160	7	6	SCO1
CmMADS22	MELO3C006159T2	Scaffold00006 1394377–1397731	6	160	4	3	SCO1
CmMADS23	MELO3C006159T4	Scaffold00006 1394377–1400874	6	221	7	6	SCO1
CmMADS24	MELO3C002049T1	Scaffold00001 891449–895415	12	258	8	7	SEP
CmMADS25	MELO3C022316T1	Scaffold00052 778977–784983	11	242	8	7	SEP
CmMADS26	MELO3C026300T1	Scaffold00090 352986–362937	N/A	246	8	7	SEP
CmMADS27	MELO3C026300T2	Scaffold00090 352986–362937	N/A	216	7	6	SEP
CmMADS28	MELO3C026300T3	Scaffold00090 354020–362937	N/A	181	6	5	SEP
CmMADS29	MELO3C007716T1	Scaffold00007 4865399–4870497	8	228	9	8	SVP
CmMADS30	MELO3C011093T1	Scaffold00014 2864281–2871142	3	230	8	7	SVP
CmMADS31	MELO3C011093T2	Scaffold00014 2864281–2871263	3	216	8	7	SVP
CmMADS32	MELO3C011093T3	Scaffold00014 2864281–2871263	3	258	9	8	SVP
CmMADS33	MELO3C011093T4	Scaffold00014 2865897–2871263	3	167	8	7	SVP
CmMADS34	MELO3C018055T1	Scaffold00031 4022390–4027175	7	218	5	4	SVP
CmMADS35	MELO3C018601T1	Scaffold00034 1382310–1411114	1	222	9	8	SVP
CmMADS36	MELO3C018601T2	Scaffold00034 1384447–1411114	1	218	8	7	SVP
CmMADS37	MELO3C002723T1	Scaffold00001 5929497–5930018	12	173	1	0	Mα
CmMADS38	MELO3C003801T1	Scaffold01596 4730952–4731518	4	188	1	0	Mα
CmMADS39	MELO3C007148T1	Scaffold00007 1127180–1128076	8	298	1	0	Mα
CmMADS40	MELO3C018030T1	Scaffold00031 3839201–3840317	7	226	2	1	Mα
CmMADS41	MELO3C020871T1	Scaffold00045 1926450–1927007	11	185	1	0	Mα
CmMADS42	MELO3C021689T1	Scaffold00049 435118–435798	12	212	1	0	Mα
CmMADS43	MELO3C023227T1	Scaffold00059 371350–372536	11	365	2	1	Mγ
CmMADS44	MELO3C009552T1	Scaffold00011 3058228–3059613	4	386	1	0	Mγ
CmMADS45	MELO3C015239T1	Scaffold00025 184813–185792	2	223	2	1	Mγ
CmMADS46	MELO3C021692T1	Scaffold00049 456773–457432	12	219	1	0	Mγ
CmMADS47	MELO3C000260T1	Contig32319 523–1093	N/A	61	2	1	Mδ
CmMADS48	MELO3C001111T1	Contig38521 110–449	N/A	86	2	1	Mδ
CmMADS49	MELO3C001991T1	Scaffold00001 531621–534860	12	364	10	9	Mδ
CmMADS50	MELO3C006159T1	Scaffold00006 1394377–1396616	6	109	2	1	Mδ
CmMADS51	MELO3C006159T3	Scaffold00006 1394377–1400874	6	120	6	5	Mδ
CmMADS52	MELO3C006860T1	Scaffold00006 6768245–6770216	6	97	2	1	Mδ
CmMADS53	MELO3C007017T1	Scaffold00007 215217–215411	8	64	1	0	Mδ
CmMADS54	MELO3C008942T1	Scaffold00010 3726274–3726758	8	102	2	1	Mδ

Continued next page

Table 1. Continued.

Gene	Gene identification	Location	Chromosome no.	Protein length (no. of amino acids)	Exons (no.)	Introns (no.)	Subfamily
CmMADS55	MELO3C011410T1	Scaffold00014 5456438–5456656	3	72	1	0	Mδ
CmMADS56	MELO3C012107T1	Scaffold00016 3060965–3064708	10	339	11	10	Mδ
CmMADS57	MELO3C022205T1	Scaffold00051 2234321–2236745	9	112	3	2	Mδ
CmMADS58	MELO3C022315T3	Scaffold00052 771547–771881	11	68	2	1	Mδ
CmMADS59	MELO3C022515T1	Scaffold00052 2277559–2277829	11	72	2	1	Mδ
CmMADS60	MELO3C026299T1	Scaffold00090 201955–202456	N/A	75	1	0	Mδ
CmMADS61	MELO3C005617T3	Scaffold00005 6501777–6505422	9	145	3	2	Mδ
CmMADS62	MELO3C005617T4	Scaffold00005 6504671–6505422	9	74	2	1	Mδ

^aChromosomal locations of these genes are not available.

Fig. 1. Chromosomal locations of melon MADS-box genes. Chr1–Chr12 represent the 12 chromosomes. The lengths of chromosomes of each MADS-box gene are displayed proportionately. Black lines on bars indicate the locations of MADS-box genes. Type I genes were not underlined and type II genes were underlined.

Furthermore, *CmMADS47* and *CmMADS48* were located on Contig32319 and Contig38521 on Scaffold00090, the chromosomal location of which remains unknown. The genomic distribution result indicated that 61% of melon MADS-box genes were clustered, and most of them presented in groups of two or more genes separated by 10 to 600 kb. It has been reported that a similar situation exists in arabidopsis, cucumber, and peach (*Prunus persica*) MADS-box genes. But the distribution of subfamily had no specificity.

We created a schematic of exon and intron structures (Fig. 2) based on the DNA and coding sequences of the genes, which showed that most type II genes had complex exon and intron structures and that there were 14 to 21 kb long introns in *CmMADS10*, *CmMADS11*, *CmMADS18*, *CmMADS35*, and *CmMADS36*. Type I genes had simpler structures, with only one exon and no intron for *CmMADS37*, *CmMADS38*, *CmMADS39*, *CmMADS41*, *CmMADS42*, *CmMADS44*, *CmMADS46*, *CmMADS53*, *CmMADS55*, and *CmMADS60*. A relatively complex exon–intron structure was only observed in *CmMADS49*, *CmMADS51*, and *CmMADS56* among type I genes; the others only had a few introns.

PHYLOGENETIC ANALYSIS OF MELON MADS-BOX GENES. To study the family relationships among the melon MADS-box genes and classify them into subfamilies, a phylogenetic tree was built after multiple sequence alignment of 62 *CmMADS*-box genes, 58 *AtMADS*-box genes, and 26 *CsMADS*-box genes using MEGA 5.1. The results corresponded with previous results for MADS-box genes. The 62 MADS-box genes of melon were divided into types I and II. There were 26 genes (*CmMADS37*–*CmMADS62*) in type I, which was further subdivided into the Mα (6 members), Mγ (4 members), Mδ (16 members), and Mβ (0 members) subfamilies (Fig. 3). Type II included 36 genes, which belonged to the MIKC^C subfamily and were distributed in 10 subclasses: SEP (5 members), AGL6 (2), AP1/FUL (2), SCO1 (5), AG (5), AGL17 (4), AGL15 (2), AP3/PI (2), SVP (8), and FLC (1); there were no genes in the AGL12, BS, TM8, and MIKC* groups (Fig. 4). Meanwhile, we collected and organized the number of MADS-box subfamily of arabidopsis, rice, peach, Chinese cabbage (*Brassica rapa*), soybean, and cucumber (Table 2), and compared them with melon. According to the statistics, the melon MADS-box gene family has roughly the same

Fig. 2. Exon-intron structures of the melon MADS-box genes. Introns and exons are represented by black lines and red solid boxes, respectively. Blue bold lines represent upstream/downstream regions; CDS = coding sequence.

characteristics as in the abovementioned species, but also shows obvious specificity. There are no genes distributing on M β and MIKC* subfamily in melon, whereas the gene number of M δ subfamily is more than other pieces.

CONSERVED MOTIF ANALYSIS. To examine the characteristics of the melon MADS-box gene family and the conserved motifs of different subfamilies, we searched for motifs via the MEME software and found 10, which we named motifs 1–10 (Fig. 5).

Fig. 3. Phylogenetic relationships of 26 type I melon MADS-box genes to their homologs in arabidopsis and cucumber. The tree was constructed using the MEGA software [version 4.1 (Tamura et al., 2007)]. The bootstrap value is 1000. Triangles indicate arabidopsis MADS-box genes, squares indicate cucumber, and dots indicate melon.

All 62 melon MADS-box proteins had motif 1, which was found in the N-terminus and is an essential conserved motif for the MADS-box gene family as it encodes the MADS-box domain. Motif 2 was recognized as the highly conserved K domain, which is found in all type II genes and is an important criterion to distinguish type I and II genes. Although the K domain was found in the *CmMADS09* and *CmMADS18* genes by SMART, it was not observed in these genes in the motif distribution diagram. Motif 4 was only found in MIKC-type genes, not in type I MADS-box genes. Motifs 7 and 8 were only found in the SVP subfamily. Motifs 6, 9, and 10 were present in the AG, My, and AGL17 subfamilies, respectively. Motifs 3 and 5 were found in both type I and II genes. The degree of motif conservation was relatively low except for motifs 1 and 2.

EXPRESSION ANALYSIS IN DIFFERENT TISSUES. To explore the expression patterns of the melon MADS-box genes in different tissues, we conducted RT-PCR analysis on the 62 MADS-box genes in roots, stems, leaves, flowers, and fruit (0 DAP), and qRT-PCR using 20 genes with tissue-specific expression (underlined in Figs. 6 and 7) in roots, stems, leaves, flowers, and fruit (0, 15, and 35 DAP). The qRT-PCR assays of expression levels in fruit at 15 and 35 DAP were performed because 15 DAP represents the melon development period and 35 DAP represents the mature period. Because *CmMADS06* shared high sequence similarity with *CmMADS07*, *CmMADS10* with *CmMADS11*, *CmMADS19* with *CmMADS20*, *CmMADS26* with *CmMADS27*, and *CmMADS50* with *CmMADS51*, the use of gene-specific primer pairs (Supplement 1) was impossible. Therefore, the transcript levels measured for these genes

Fig. 4. Phylogenetic relationships of 36 type II melon MADS-box genes to their homologs in Arabidopsis and cucumber. The tree was constructed using the MEGA software [version 4.1 (Tamura et al., 2007)]. The bootstrap value is 1000. Triangles indicate Arabidopsis MADS-box genes, squares indicate cucumber, and dots indicate melon.

Table 2. Numbers of MADS-box subfamily in seven species. The MADS-box genes were divided into type I and II genes. Type I genes contained M α , M β , M γ , and M δ subfamilies. Type II genes contained MIKCC and MIKC* subfamilies (Henschel et al., 2002; Pařenicov et al., 2003).

Species	Subfamilies (no.)						Total (no.)
	Type I				Type II		
	Mα	Mβ	Mγ	Mδ	MIKC ^C	MIKC*	
<i>Arabidopsis thaliana</i> (Pařenicov et al., 2003)	20	17	21	4	43	2	107
<i>Oryza sativa</i> (Arora et al., 2007)	13	9	10	0	38	5	75
<i>Prunus persica</i> (Wells et al., 2015)	21	7	12	0	35	4	79
<i>Brassica rapa</i> (Duan et al., 2014)	27	16	22	0	84	11	160
<i>Glycine max</i> (Shu et al., 2013)	18	5	11	0	67	5	106
<i>Cucumis sativus</i> (Hu et al., 2012)	5	2	3	3	29	1	43
<i>Cucumis melo</i>	6	0	4	16	36	0	62

were only indicative of the mixed transcript level. The gene expression patterns within each group were often conserved to some extent, although the expression levels of specific

members varied in different organs. The RT-PCR and qRT-PCR analyses showed that the expression levels of AG subclass genes, which were specifically expressed in fruit, gradually declined during fruit development and ripening, although the expression level of *CmMADS04* was too low to show this. Only one gene (*CmMADS06*) of the AGL6 subfamily was specifically expressed in flowers and fruit (0 DAP) (Fig. 6). This gene had extremely low or no expression in different developmental stages of other tissues or fruit. In the API/FUL subfamily, *CmMADS14* and *CmMADS15* were specifically expressed in flowers and fruit (Fig. 6), and the transcript level of *CmMADS14* during the fruit development stage was relatively high (Fig. 8).

Fig. 5. Conserved motifs of melon MADS-box proteins identified using the MEME program. Motifs 1 to 10 are indicated by different colored boxes. The names of all members and combined probability values are shown on the left side; motif sizes are shown at the bottom. The order of the motifs corresponds to their positions in individual protein sequences.

Although *CmMADS16* had low expression in leaves and fruit, the AP3/PI subfamily showed specific expression in flowers. *CmMADS17* showed an extremely high expression level in flowers (Fig. 8). Most SEP subfamily genes were specifically expressed in flowers and fruit. During fruit development,

CmMADS25 showed rapid downregulation of expression and *CmMADS26* was upregulated and maintained a high transcript level, even entering into the mature period. The expression of AGL17 subclass genes was specific to and high in roots. The expression levels of SVP subclass genes were different in

Fig. 6. Expression analysis of type II melon MADS-box genes in different tissues using reverse-transcription polymerase chain reaction (RT-PCR). RT-PCR was performed using primers specific to each gene. PCR products were run on 2% agarose gels. *CmACTIN* was used as an internal standard for each gene. The roots, stems, leaf, flowers, and fruit at 0 d after pollination were collected from melon plant at the flowering stage. The expressions of the underlined genes were analyzed by quantitative real-time RT-PCR.

Fig. 7. Expression analysis of type I melon MADS-box genes in different tissues using reverse-transcription polymerase chain reaction (RT-PCR). RT-PCR was performed using primers specific to each gene. PCR products were run on 2% agarose gels. *CmACTIN* was used as an internal standard for each gene. The roots, stems, leaf, flowers, and fruit at 0 d after pollination were collected from melon plant at the flowering stage. The expressions of the underlined genes were analyzed by quantitative real-time RT-PCR.

different tissues, being highest in leaves and hardly detectable in fruit (Figs. 6 and 8). Among type I genes, the expression of M α and M γ subfamily members was low (Fig. 7), but relative quantitative results showed that M α *MADS37* and *MADS40* had relative high expression in fruit of 35 DAP and flowers (Fig. 8). The M δ subfamily genes showed diverse expression in different tissues, such as a high expression levels for *CmMADS47*, *CmMADS58*, and *CmMADS59* in fruit (35 DAP), fruit (0 DAP), and roots, respectively (Fig. 8). *CmMADS47* was specifically expressed in flower and the different stage of fruit (Fig. 7), and the expression level was highest at the mature fruit (Fig. 8).

Discussion

In recent years, with the rise and development of sequencing techniques, bioinformatics has rapidly expanded.

Bioinformatics-based prediction and detailed studies of the MADS-box gene family have been performed in many species. In this study, we identified and analyzed the MADS-box gene family in melon, finding 62 MADS-box genes. There are more MIKC^C-type genes than MIKC*-type genes in some plant species, such as arabidopsis, cucumber, and apple, whereas MIKC*-type genes are not found in melon. Previous research has shown that MIKC*-type MADS-box genes have a large influence on pollen viability. MIKC*-type gene mutations lead to extremely reduced pollen fecundity, because of reduced pollen viability, delayed germination, and aberrant pollen tube growth (Adamczyk and Fernandez, 2009). So other genes in melon could regulate the pollen viability and fecundity. Type I MADS-box genes are divided into the M α , M β , M γ , and M δ subfamilies, and have a diverse distribution in the representative species mentioned above.

Fig. 8. Expression analysis of selected melon MADS-box genes in different tissues and three fruit development stages using quantitative reverse-transcription polymerase chain reaction. *CmACTIN* was used as an internal standard for each gene. The mean expression value was calculated from nine independent replicates. The vertical bars indicate the standard deviation. Fruit at 0 d after pollination [DAP (Fr1)], fruit at 15 DAP (Fr2), and fruit at 35 DAP (Fr3).

Generally, few or no genes are distributed in Mδ. We found no Mβ subfamily genes in melon, which means that Mβ subfamily genes are not essential for melon or that their functions have been replaced by other genes. Furthermore, we found 16 members in the Mδ subfamily, whose ratio (61.5%) in type I was higher than in the above mentioned species. We know little about the functions of type I MADS-box genes, so the significance of the distribution of these two subfamilies remains unclear. In arabidopsis, the AGL12 gene is closely related with the development of roots (Tapia-López et al., 2008), and overexpression of this gene in rice not only influences root development, but also induces chlorosis, cell death, and pigment accumulation (Lee et al., 2008). However, we did not find any AGL12 subclass genes in melon, suggesting root

development and pigment accumulation are not regulated by AGL12 subclass genes in melon. The TT16 gene in arabidopsis, belonging to the BS group, has a confirmed correlation with seed coloring (Nesi et al., 2002). No BS subfamily genes were found in melon, which may be related to the tendency of melon seeds to be colorless. Similarly, the AGL12 and BS subgroups are not found in cucumber (Hu et al., 2012), which has a close genetic relationship with melon, highlighting the affinity between these two species.

Our exon-intron structure analysis showed that melon type I MADS-box genes were relatively short and had simple structures, but MIKC-type genes had a complex structure with short or long introns. The Mα and Mγ genes usually had no intron or a single intron, whereas MIKC genes and several

M δ genes contained multiple introns. The similar situation existed in arabidopsis, rice, cucumber, and apple (*Malus $\times$ domestica*) (Arora et al., 2007; Hu et al., 2012; Pařenicová et al., 2003; Tian et al., 2015). These results suggested that the structure of MADS-box genes was conservative relatively in these species.

Previous studies have shown that MADS-box genes of diverse subfamilies have different expression patterns in different tissues of arabidopsis, grape, apple, and cucumber. For example, AP3/PI subclass genes are specifically expressed in grape flowers, cucumber fruit, and apple stems, leaves, and flowers, whereas their expression was specific to and high in melon flowers. It is reported that the AP3/PI genes controlled the formation of petals and stamens during arabidopsis flower development, and might act as a switch between the activation of male and the repression of female development (Wuest et al., 2012). Thus, their expression suggested a role in melon flower development. AGL6 subclass genes have been found to be specifically expressed in cucumber fruit and grape flowers, whereas their expression was high in melon flowers and fruit. AGL17 genes are expressed at different levels in diverse tissues and are specifically expressed in grape fruit and cucumber roots; our results in melon were consistent with those in cucumber, with specific expression in roots. In melon, the expression levels of M α and M γ subfamily genes were low, and some was even undetectable in roots, stems, flowers, and fruit at 0, 15, and 35 DAP. But a low transcript level of *MADS37* and *MADS38* was detected in fruit at 0 DAP, and *MADS40* in flowers. Quantitative results showed that *MADS40* was expressed specifically in flowers, and the relative expression levels are about 170 times than other tissues. We infer that genes of both of these subfamilies are pseudo genes, or are expressed only in specific tissues or at a specific stage. SEP-like genes are expressed in a range of species with fleshy fruits, including tomato (*Solanum lycopersicum*), peach, strawberry (*Fragaria $\times$ ananassa*), and so on. It was highly expressed during fruit ripening in tomato and strawberry, and suppression of the gene delayed normal fruit ripening (Seymour et al., 2011; Vrebalov et al., 2002). In this study, the expression of SEP-like gene *CmMADS26* increased gradually with the mature of melon fruit. Hence, it is tempting to speculate that the gene *CmMADS26* may play similar roles in melon. These data generated here will be useful for analysis of the biological functions of MADS-box family genes in melon growth and development.

In brief, 62 MADS-box genes in melon distributed across its 12 chromosomes, often located within the same small chromosomal region in clusters of two or more genes. Phylogenetic comparisons and expression analysis in this study will provide information for understanding the classification, cloning, and predicting biological functions of the family in melon.

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