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Identification and expression analysis of seven MADS-box genes from *Annona squamosa*

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Abstract

MADS-box genes encode a family of transcription factors that regulate diverse growth and developmental processes in plants, including flowering. In this study, comprehensive characterization and expression profiling analyses of seven sugar apple (*Annona squamosa* L.) MADS-box genes were performed using rapid amplification of cDNA ends method. Domain and phylogenetic analyses grouped these seven MADS-box genes into six different clades and they showed high similarity with orthologs in *Arabidopsis*. Expression patterns of these MADS-box genes were investigated during different flower developmental stages and in various reproductive organs, including petal, stamen, sepal, and pistil. Most of the MADS-box genes studied were least expressed in the sepal and *AsAGL67* and *AsAGL80* expression was weak in all tissues. *AsSEP1* and *AsAGAMOUS* showed highest expressions in the stamen and pistil, and *AsAGL12* showed stamen-specific expression. Dynamic expression patterns of MADS-box genes in different reproductive stages suggest involvement in flower development. Interestingly, a number of these MADS-box genes showed responses to gibberellin, abscisic acid, and salicylic acid treatments, suggesting control of their expression by phytohormones.

Additional key words: abscisic acid, flower organs, flowering time, gibberellin, salicylic acid, sugar apple.

Introduction

MADS-box genes encode important transcription factors involved in development and signal transduction in eukaryotes (Ng and Yanofsky 2001, Duan *et al.* 2015) such as fungi (Passmore *et al.* 1988), animals (Norman *et al.* 1988), and plants (Sommer *et al.* 1990, Yanofsky *et al.* 1990). Eukaryotes possess a conserved DNA-binding domain, the MADS domain, which takes its name from *MCM1* (in yeast), *AGAMOUS* (in *A. thaliana*), *DEFICIENS* (in *Antirrhinum majus*), and SRF (in *Homo sapiens*) (Passmore *et al.* 1988, Sommer *et al.* 1990, Yanofsky *et al.* 1990, Pelaz *et al.* 2001). MADS-box genes are characterized by a conserved domain of approximately 58 - 60 amino acids located in the N-terminal region; this domain is involved in DNA binding and dimerization at consensus recognition sequences known as CArG boxes [CC(A/T)6GG] (Yanofsky *et al.* 1990).

Phylogenetic studies have divided the MADS-box gene family into two categories, type I and II, which originated by ancestral gene duplication. Members of type I, including SRF-like genes, are found in animals, fungi, and plants; type II includes MEF2-like genes, found in animals and yeast, as well as plant-specific MIKC-type MADS-box genes (Alvarez-Buylla *et al.* 2000, De Bodt *et al.* 2003, Xu *et al.* 2014, Duan *et al.* 2015, Wells *et al.* 2015). MIKC-type proteins generally have four common domains. In addition to a MADS domain (M), MIKC-type proteins contain intervening (I), keratin-like (K), and C-terminal (C) domains (Norman *et al.* 1988, Cho *et al.* 1999, Yang *et al.* 2003. Kaufmann *et al.* 2005, Smaczniak *et al.* 2012). The K domain is a conserved region characterized by a coiled-coil structure, which contributes to the dimerization of MADS-box proteins. The K domain, present in type II but absent in

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Abbreviations: ABA - abscisic acid, AG - AGAMOUS; CaMV - cauliflower mosaic virus; GA - gibberellin; GFP - green fluorescent protein; NJ - neighbour-joining; RACE - rapid amplification of cDNA ends; SA - salicylic acid.
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type I proteins, is more highly conserved (Diaz-Riquelme *et al.* 2009, Shu *et al.* 2013).

MADS-box genes play a central role in many important biological processes in plants, including flowering (*i.e.*, *SOC1*, *FLC*, *SVP, AGL24*, and *MAF1/FLM*), floral organ identity (*AP1*, *FUL*, and *AG*), floral morphogenesis (*AP1*, *SEP1-3*, *AP3*, *PI*, and *AG*), fruit development (*SHP1* and *SHP2*), and embryo development (*TT16*) (Dornelas *et al.* 2010, Masiero *et al.* 2011, Li *et al.* 2012a, Shu *et al.* 2013). In some flowering plants, MADS-box genes work as organ identity genes during flower development, and loss-of-function mutations in these genes cause homeotic transformations of floral organs (Schwarz-Sommer *et al.* 1990). Homeotic floral mutant analysis has contributed to the formulation of a genetic model 'ABCDE', which explains how the combined functions of five gene classes (A - E) determine the identity of the four flower organs. In this well-known model, except for class A genes, such as *APETALA2* in *Arabidopsis*, all floral organs identity genes A, B, C, D, and E have a highly conserved MADS-box domain, and thus belong to the MADS-box gene family (Lu *et al.* 2012).

Plant growth and development are influenced by endogenous regulators and environmental factors (Duan *et al.* 2015). Gibberellin (GA) regulates growth of plant organs by promotion of cell division and elongation (Little and MacDonald, 2003). In addition, GA promotes flower formation and flowering time in some species. Its involvement in flower initiation in plants is well established, and there is growing insight into the mechanisms by which floral induction is achieved (Sakamoto et al. 2001, Mutasa-Göttgens and Hedden 2009, Li *et al.* 2012b). Abscisic acid (ABA) regulates many aspects of plant growth and development (Bezerra *et al.* 2004, Wilmowicz *et al.* 2008). Salicylic acid (SA) also regulates flowering time, as SA-deficient plants are late flowering (Martínez *et al.* 2004).

Sugar apple (*Annona squamosa* L.), a commercially important fruit tree, is commonly grown in tropical and subtropical regions worldwide. Sugar apple is semi-deciduous tree that progressively sheds its leaves in the spring. Compared to *Arabidopsis*, rice, and maize, relatively fewer MADS-box genes have been characterized in woody plants. The molecular characteristics of sugar apple MADS-box genes and their functions are still unknown. Despite what is known about phytohormone involvement in plants, there is little understanding of how MADS-box genes in sugar apple respond to hormone signals during flowering. The aim of this study was to isolate seven MADS-box genes from sugar apple for molecular characterization by sequence and phylogenetic analysis, expression profiling, and subcellular localization, in order to better understand the genetic control of flowering in fruit trees and, more broadly, in plants in general.

Materials and methods

Plants and growth conditions: 12-year-old sugar apple (*Annona squamosa* L.) trees were planted in a 4 × 4 m plots with drip irrigation at the Lingnan Normal University field experimental station in Zhanjiang City, Guangdong Province, China. Agronomic practices and fertilizer applications were applied as needed (Liu *et al.* 2015). The area has a tropical oceanic monsoon climate with an average daily temperature of 22.8 °C, a minimum temperature of 15.7 °C, and a maximum temperature of 28.8 °C. The total yearly rainfall is in the range of 1 100 to 1 800 mm.

Isolation and identification of MADS-box genes: Polymerase chain reaction (PCR) using a *SMART*™ rapid amplification of cDNA ends (RACE) cDNA amplification kit (*Clontech*, Mountain View, CA, USA) was performed according to the manufacturer′s instructions. The sequences of the conserved MADS-box domain from *Arabidopsis* were used for gene specific primer design. Total RNA of sugar apple plant (1 μg) was used for 5′-cDNA synthesis. After the 5′-sequence of each MADS-box gene sequence was determined, the full-length cDNA was isolated by a second round of reverse transcription (RT)-PCR. Primers are listed in Table 1 Suppl. PCR conditions included five cycles of initial denaturation at 94 °C for 30 s followed by annealing at 72 °C for 3 min; five cycles of denaturation at 94 °C for 30 s, annealing at 70 °C for 30 s, extension at 72 °C for 3 min; and 27 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s, and a final extension at 72 °C 3 min. PCR products were separated on a 1 % agarose gel and the single specific band of PCR products was cloned into the pMD18-T vector (*TaKaRa*, Dalian, China) for sequencing.

Protein tertiary structure characterization and phylogenetic analyses: Multiple sequence alignments were created for the seven sugar apple MADS-box protein sequences using the *ClustalW* software with default parameters. Using the *pfam* database, the accession ID of the K-box domain was determined as pfam01486. All MADS-box protein sequences were determined using *BLAST* on NCBI. Phylogenetic trees were constructed from MADS-box protein sequence alignments by seven sugar apple and 108 *Arabidopsis* sequences with *MEGA v. 5.1* (http://www.megasoftware. net/mega5/mega.html) by the neighbor-joining (NJ) method. Bootstrap values were calculated from 1 000 iterations. Information on MADS-box genes in *Arabidopsis* is provided in Table 2 Suppl. Sugar apple MADS-box genes were named according to relationship homology in the NJ tree.

Construction of expression vectors and subcellular localization analysis: Full-length coding regions of seven sugar apple MADS-box cDNA sequences were cloned into the vector pH7FWG2.0 to generate expression constructs. An artificial green fluorescent protein (GFP), fused in-frame to the C terminus of each MADS-box protein, was placed under the control of a cauliflower mosaic virus (CaMV) 35S promoter. The 35S:GFP vector was used as a negative control. These constructs were transiently expressed in tobacco (*Nicotiana benthamiana*) epidermal cells using *Agrobacterium* transformation. Fluorescence of fusion protein constructs was detected using a confocal microscope *LSM710* (*Carl Zeiss*, Oberkochen, Germany).

RNA isolation and RT-qPCR: Total RNA from different tissues, including root, stem, leaf, flower, and fruit, was extracted using a *Plant RNeasy* mini kit (*Qiagen*, Hilden, Germany) according to the manufacturer's instruction. DNase I was used to remove any genomic DNA contamination from total RNA. For each sample, cDNA was synthesized from 3.0 μg total RNA by *SuperRT* reverse transcriptase (*CoWin Biotech*, Beijing, China) using oligo (dT) primers. The primers sequences of RT-qPCR were designed using the *Premier 5* software (*Premier Biosoft International*, Palo Alto, CA, USA). *AsActin* was used as an internal standard to calculate relative fold differences based on comparative cycle threshold ($2^{-\Delta\Delta Ct}$) values. Primer sequences for *AsActin* were as follows: forward 5′-GACACCATC CCCAGAATCC-3′ and reverse 5′-CCCCAGAAGAAC ACCCTGT-3′. The RT-qPCR procedure was performed as follows: 1 mm^3 of a 1/10 dilution of cDNA in ddH_2O was added to 5 mm^3 of 2× *SYBR® Green* buffer, 0.1 μM of each primer, and ddH_2O to a final volume of 10 mm^3. All primer sequences for sugar apple MADS-box genes are listed in Table 3 Suppl. Heat map representation was performed using the average Ct value with the software *MeV* (*v.4.9*) to visualize and cluster RT-qPCR data based on the HCl method (Eisen *et al.* 1998).

Standard curves and efficiency of amplification: The limit of detection and the amplification efficiency of the RT-qPCR were determined using a 10-fold serial dilution of cDNA isolated from leaves, which was used to create the standard curve. The slopes and correlation coefficients of the standard curves were used to calculate the PCR efficiency (E) of primer pairs. In our experiment, the E value of each primer pair was calculated by formula: E = POWER (10, 1/slope) - 1. The E value for each primer pair was between 0.9 and 1.1 (Table 3 Suppl.). Then, the invariant expression of several housekeeping genes under different experimental conditions was tested. In our study, 1 mm^3 of cDNA (30 ng mm^{-3}) from different experimental samples were used as templates for RT-qPCR analysis (Fig. 1 Suppl.). Based on the transcriptome data, several housekeeping genes, including *Actin*, *GAPDH*, *UBQ*, and *18SrRNA*, were used for reference gene selection. The stability values of these housekeeping genes were calculated using the *NormFinder* software (http://moma.dk/normfinder-software/). Because of the lowest stability value, the *Actin* gene has been selected as a reference.

Flower collection under different developmental stages: In this experiment, flowers of six different developmental stages were collected, including three stages of flower buds based on size (diameter 1, 3, and 7 mm used as Stage I, II, and III, respectively), young flowers with closed petals (Stage IV), mature flowers with partially opened petals (stage V), and mature flowers with opened petals (stage VI). All samples were frozen in liquid nitrogen and stored at -80 °C for further analyses. Plant tissues used for the expression analyses included petal, stamen, sepal, and pistil of mature flowers (stage VI).

Hormone treatments: The flower buds (1 mm diameter) of 10- to 12-year-old sugar apple trees were cultured under four treatments: control, 100 μM ABA, 100 μM GA, and 100 μM SA. At 2, 4, and 12 h post-treatment, the flower bud samples were collected, frozen in liquid nitrogen, and stored at -80 °C for further analysis.

Statistical analysis: Significant differences between values were calculated using a one-way analysis of variance with Student's *t*-test at a significance level of $\alpha = 0.01$ in the *Excel* software. All expression analyses were performed for five biological replicates. All reported values represent arithmetic means of five replicates, and data are expressed as mean ± SD.

Results

Based on the conserved MADS-box domain from *Arabidopsis*, seven full-length cDNA sequences were identified as putative candidate sugar apple MADS-box genes for further analyses. A phylogenetic tree, built to explore the evolutionary relationship of MADS-box genes between sugar apple and *Arabidopsis*, showed genes homologous to *Arabidopsis* MADS-box sequences in the genome of sugar apple (Fig. 1). In sugar apple, two genes, *AsSEP1* and *AsSEP3*, were grouped into the SEP subfamily and are homologous to *AtSEP1* and *AtSEP3*, respectively. *AsAGAMOUS* and *AsAGL12, 15, 67, 80* showed homology to *AtAGAMOUS* and *AtAGL12, 15, 67, 80,* respectively. Gene names, GenBank accession numbers, subcellular locations, predicted polypeptide

lengths, molecular masses, and subfamily classifications are summarized in Table 4 Suppl. Sizes of the deduced MADS-box proteins ranged from 198 (AsAGL12) to 337 amino acids (AsAGL67), molecular masses ranged from 22.36 to 37.76 kDa, and predicted isoelectric points ranged from 5.79 (AsAGL67) to 9.41 (AsAGL80).

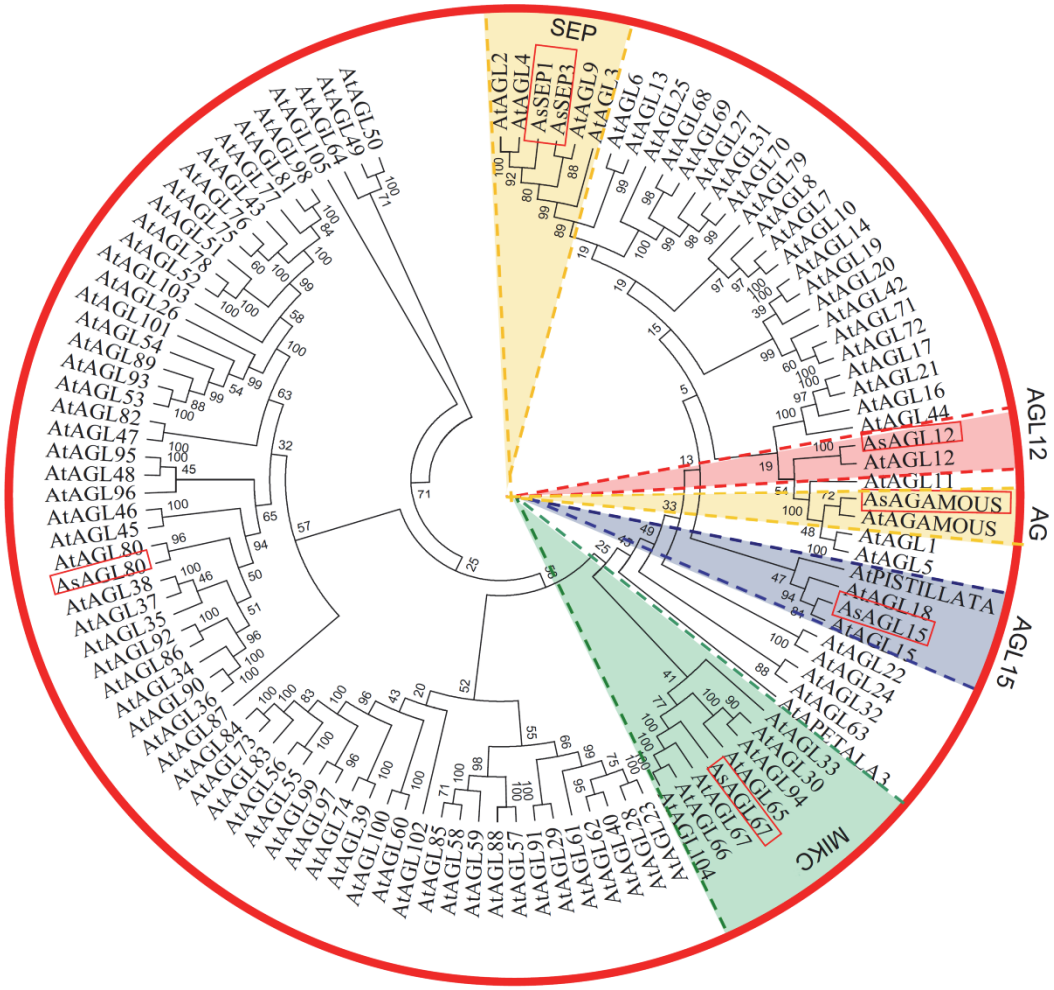

Fig. 1. The phylogenetic relationship of MADS-box genes between sugar apple and *Arabidopsis*. An unrooted phylogenetic tree constructed using sequences of seven sugar apple MADS-box protein and Type II MADS-box proteins from *Arabidopsis*. The neighbor-joining model with pairwise deletion was selected. *Colour dashed lines* indicate the sugar apple amino acid proteins belonging to the different subfamilies. *Red boxes* highlight sugar apple proteins.

Multiple sequence alignments were created for deduced polypeptides of seven sugar apple MADS-box proteins, and their similarities are shown in Fig. 2 Suppl. Five of the MADS-box proteins contained a classical MADS-box domain and a K-box domain. Two other MADS-box proteins, AsAGL15 and AsAGL80, contained only a MADS-box domain. Furthermore, we investigated the predicted 3D structures of these seven MADS-box proteins using *Phyre2* (http://www.sbg.bio.ic.ac.uk/phyre2/), and this indicated that all seven MADS-box proteins share a similar 3D structure (Fig. 2). Interestingly, there were some small differences among different types of MADS-box proteins. Only five α-helices were contained in the N-terminus of AsAGAMOUS and AsAGL80, while all other MADS-box proteins contained six α-helices.

According to previous reports in other species, most MADS-box proteins are localized to the nucleus (Zhang *et al*. 2012). We observed transient expression of seven cloned MADS-box proteins in the epidermal cells of *N. benthamiana* leaves, indicating that sugar apple MADS-box proteins localize to the nucleus (Fig. 3).

In this study, real time qPCR was used to examine tissue-specific expression patterns of seven sugar apple MADS-box genes in different tissues and organs, including roots, stems, leaves, flowers, and fruits. An accumulation of MADS-box mRNA was detected in at least one tissue or organ for every genotype (Fig. 4). The transcriptions of *AsAGL67, AsAGL12,* and *AsAGAMOUS* were barely detectable in leaves, suggesting limited roles in leaf growth and development. *AsAGL12* was highly expressed in root tissue, which suggests that it may play

an important role in root elongation. Interestingly, most of the MADS-box genes tested in this experiment were highly expressed in flowers and fruits.

To determine the roles of MADS-box genes in flower development and maturation, expression patterns of seven MADS-box genes were investigated during six develop-

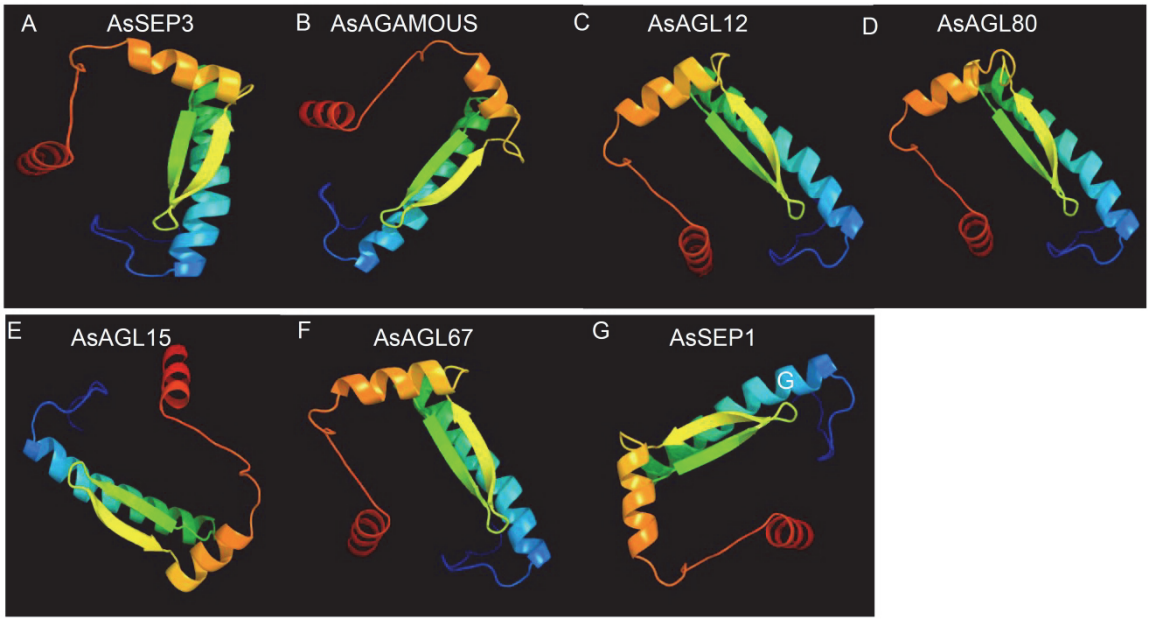

Fig. 2. Tertiary structure predictions of four MADS-box proteins in sugar apple. Three-dimensional structure of consensus sequences of seven MADS-box proteins, including AsSEP3 (*A*), AsAGAMOUS (*B*), AsAGL12 (*C*), AsAGL80 (*D*), AsAGL15 (*E*), AsAGL67 (*F*), and AsSEP1 (*G*). Different coloured stands represent different α-helices and β-sheets. The online software *PyMol* (http://www.pymol.org/) was used to predict the 3D structures of several MADS-box proteins in sugar apple.

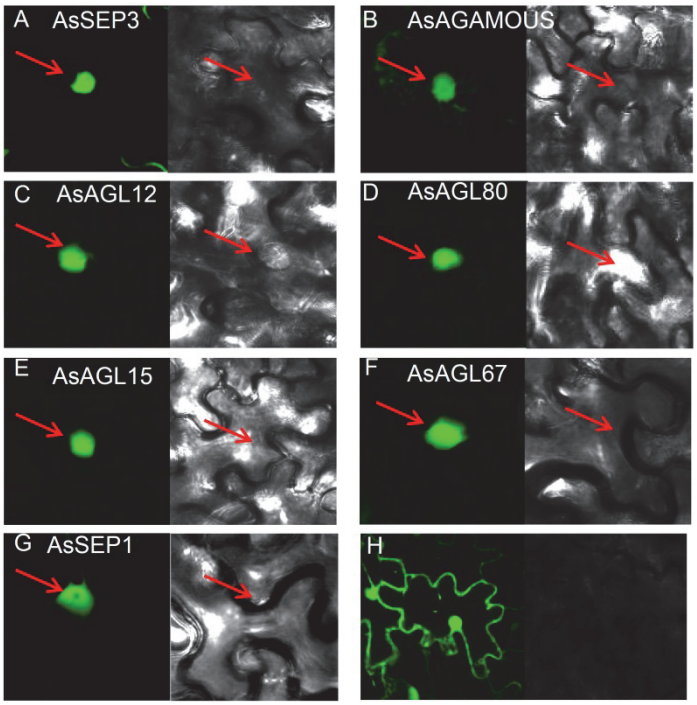

Fig. 3. Subcellular localization of sugar apple MADS-box proteins. MADS-box gene-GFP fusion constructs transiently expressed in tobacco epidermis cells (green fluorescence of MADS-box gene-GFP and bright-field): AsSEP3 (*A*), AsAGAMOUS (*B*), AsAGL12 (*C*), AsAGL80 (*D*), AsAGL15 (*E*), AsAGL67 (*F*), and AsSEP1 (*G*). The 35S: GFP vector was used as negative control (*H*).

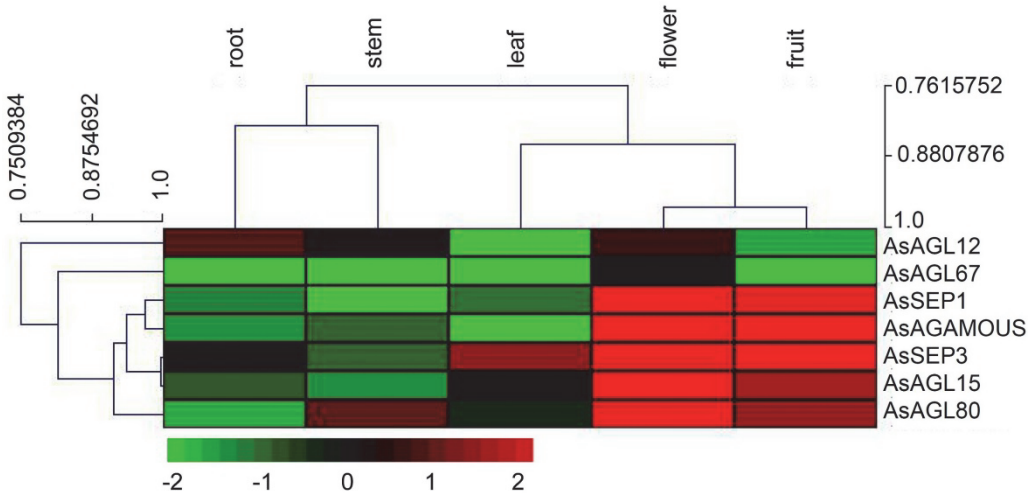

Fig. 4. Expression patterns of seven MADS-box genes in different tissues, including roots, stems, leaves, flowers, and fruits of sugar apple plants. Means are from five independent replicates. Expression levels are shown on a log2 scale from high to low for each MADS-box gene. The different colours correspond to the log-transcription values of the gene change-fold ratio shown in the bar.

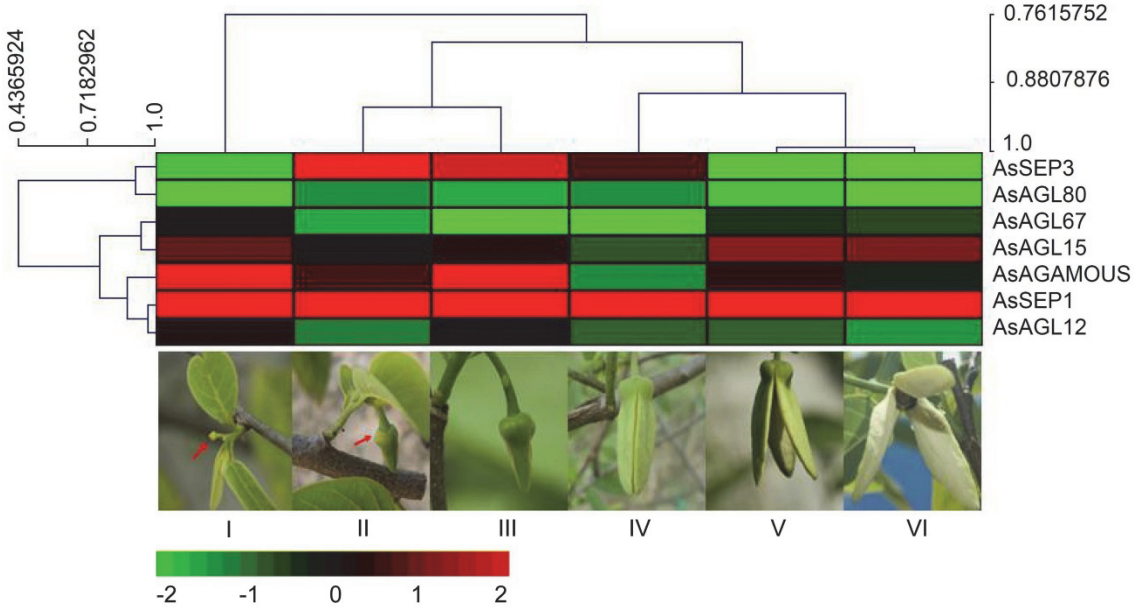

Fig. 5. A heatmap showing seven sugar apple MADS-box gene expressions in flowers during different developmental stages (shown below). Levels of down-regulated (*green*) and up-regulated (*red*) expression are shown on a log2 scale from high to low for each MADS-box gene. The different colours correspond to the log-transcription values of the gene change-fold ratio shown in the bar. In this experiment, flowers of six different developmental stages were collected, including three stages of flower buds based on size (diameter 1, 3, and 7 mm used as Stage I, II, and III, respectively), young flowers with closed petals (Stage IV), mature flowers with partially opened petals (stage V), and mature flowers with opened petals (stage VI).

mental stages. Except for *AsAGL80* that demonstrated high expression across all flowering stages, the remaining MADS-box genes exhibited more dynamic expression patterns. In addition, expression patterns of MADS-box genes belonging to the same phylogenetic subfamily also varied significantly. *AsSEP3* showed a peak expression in stage II and a significant decrease in expression at maturation (Fig. 5). In contrast, changes in expression of *AsSEP1* were undetectable during the developmental process.

To elucidate their function in flower morphology, we analyzed the expression of these MADS-box genes in different flower organs, including petals, stamens, sepals, and pistils from the same collection of flowers. Expression of *AsAGL67* and *AsAGL80* was weak in all organs. Most MADS-box genes demonstrated the lowest expressions in the sepal. *AsSEP1* and *AsAGAMOUS* displayed the highest expressions in both stamens and pistils; *AsAGL12*, showed stamen-specific expression (Fig. 6).

During growth and development, plants are influenced by numerous growth regulators. To identify the putative roles of MADS-box genes under GA, ABA, and SA treatments, we determined comprehensive expression profiles of seven MADS-box genes. Expressions of most genes were upregulated, but some were down-regulated or showed no change under these treatments (Fig. 7). Under GA treatment, *AsAGL67* and *AsAGL80* were upregulated at 2 and 4 h. They reached the highest expression at 4 h. In contrast, three genes (*AsAGAMOUS*, *AsAGL12*, and *AsAGL15*) were down-regulated at 2 and 4 h. *AsSEP1* and *AsSEP2* showed no response to GA (Fig. 7).

Under ABA treatment, *AsAGL12* and *AsAGL15* had

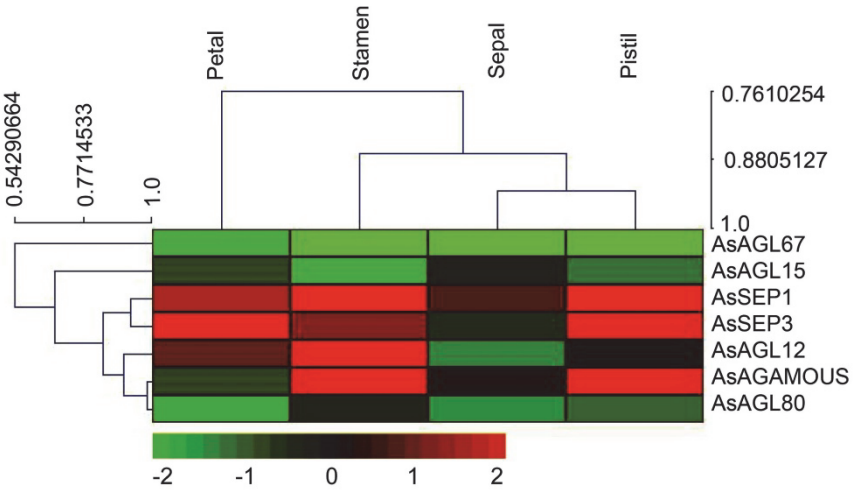

Fig. 6. Changes in expression of sugar apple MADS-box genes in different floral organs including petal, stamen, sepal, and pistil Levels of expression are shown on a log2 scale from high to the low of each MADS-box gene.

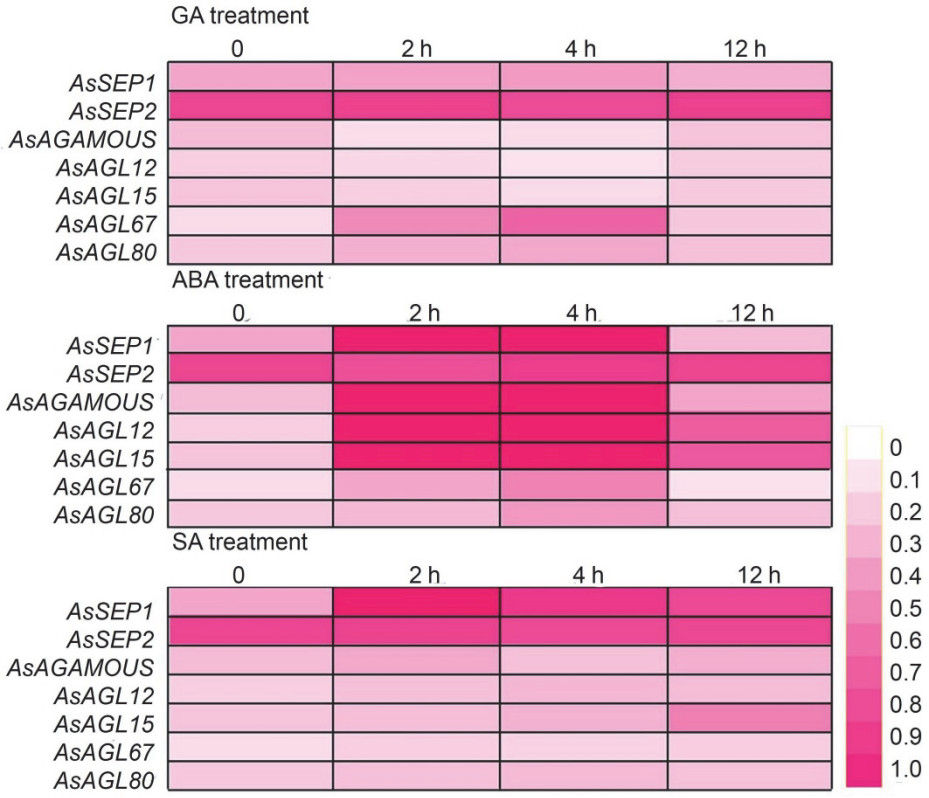

Fig. 7. Expression analysis of sugar apple MADS-box genes under GA, ABA, and SA treatments. Every treatment contain three times (2, 4, and 12 h). The relative expression levels of MADS-box genes in flower under these treatments were quantified against the control transcription levels. The *bar* at the bottom of each heat map represents relative expression values.

high expressions at 2 h, but were down-regulated at 12 h. The expression of these two genes were over 20 times that of the control at 2 h. *AsSEP1* and *AsAGAMOUS* reached their highest expressions at 2 h, but they were down-regulated at 12 h. *AsAGL67* reached its highest expression at 4, and it was reduced at 12 h. *AsSEP2* and *AsAGL67* showed no significant responses to ABA treatment (Fig. 7).

AsSEP1 was upregulated under SA treatment; however, the remaining genes (*AsSEP3*, *AsAGAMOUS*, *AsAGL12*, and *AsAGL67*) showed no significant responses to SA treatment (Fig. 7).

Discussion

MADS-box genes encode transcription factors that play important roles in regulating the processes of development and signal transduction in eukaryotic organisms (Theissen *et al.* 1996, 2000, Zhang *et al.* 2012). In flowering plants, some MADS-box genes are involved in the transition to reproduction and the development of flowers (Schwarz-Sommer *et al.* 1990).

A well-known MADS-box protein, AGAMOUS, has been reported to be involved in flower development and seed morphology (Groth *et al.* 2011, Tanaka *et al.* 2013, Almeida *et al.* 2015). Recently, several SEPALLATA-like subfamily MADS-box genes play roles in flower development (Pelaz *et al.* 2001, Huang *et al.* 2009). Here, we identified one homolog of *AtAGAMOUS* (*AsAGAMOUS*), and two homolog of *AtSEP1* (*AsSEP1* and *AsSEP3*) in sugar apple (Fig. 1). Detailed studies of these MADS-box genes may provide clues to the mechanism of flower development in sugar apple.

The study of floral homeotic mutants in model dicot species has elucidated the genetic and molecular mechanisms of flower development through the 'ABCDE' model (Weigel and Meyerowitz 1994). In angiosperms, *AGAMOUS* (*AG*) is required for floral meristem determination and performs the C-class function of stamen and carpel specification (Smaczniak *et al.* 2012). *AsAGAMOUS* in sugar apple showed homology to *AtAGAMOUS* in *Arabidopsis*, and was highly expressed in flower and fruit. In fact, *AG* in *Arabidopsis* is known to activate the expression of several downstream genes in a region of the floral meristem (Sun *et al*. 2009). *AsAGAMOUS* displays the highest expression in the stamen and pistil, similar to that observed in the ectopic expression of dicot *AG* or its homologs. It is suggested that *AsAGMOUS* may play a role in maintaining the floral meristem.

The partially redundant E-class genes *SEP1-3* assist in the formation of higher-order complexes among other floral homeotic MADS-box proteins (Smaczniak *et al.* 2012). Their orthologs in strawberry and peach also function in fruit development and ripening (Seymour *et al.* 2011, Wells *et al.* 2015). We isolated two homologous genes of *AtSEP1* (*AsSEP1* and *AsSEP3*), both of which were highly expressed in flowers and fruits and showed little expression elsewhere, suggesting their potential role during flower and fruit development. Moreover, SEP proteins act as protein connectors supporting multimeric complexes with A, B, and C proteins to activate downstream genes. Thus, *SEP* function is essential for the development of all floral organ types: sepals, petals, stamens, and carpels (Robles and Pelaz 2005). *Arabidopsis* triple-mutant lacking activity of all three *SEP* genes produce flowers containing only sepals, suggesting that SEP1-3 are required for petal, stamen, and carpel development (Pelaz *et al*. 2000). In accordance with this, *AsSEP1* and *AsSEP3* showed a floral organ-specific expression pattern, and provided clues to the functions of *SEP* family genes in sugar apple.

AGL12 constitutes its own subfamily in *Arabidopsis* and is highly expressed in roots, where it influences root meristem proliferation through effects on auxin and cell cycle regulation (Alvarez-Buylla *et al.* 2000, Tapia-Lopez *et al.* 2008). *AGL12* has been implicated in regulation of the floral transition, and its rice ortholog plays a role in stress response (Lee *et al.* 2008, Tapia-Lopez *et al.* 2008). The sugar apple ortholog, *AsAGL12*, was almost exclusively expressed in roots and flowers. MADS-box genes have received attention for their role in floral development; however, they appear to have multiple, less appreciated functions in roots.

Under the control of a strong constitutive promoter, the *AGL15* gene increases the longevity of the sepals and petals, and delays a selected set of age-dependent developmental processes, including the transition to flowering and fruit maturation in *Arabidopsis* (Fernandez *et al.* 2000). However, we observed little expression in sepals and petals in sugar apple (Fig. 6). Likewise, *AGL15* was shown to accumulate transiently in floral buds of *Arabidopsis* (Fernandez *et al.* 2000). Similarly, our findings show high expressions of sugar apple MADS-box genes in floral buds. Lastly, *AsAGL80* showed high expression in flowers and fruits, suggesting that it may play an important role in flower and fruit development.

Genetic controls of flowering time and flower development are important for the successful breeding of sugar apple. As important regulators of both flowering and flower development, these MADS-box genes are key factors to the breeding of sugar apple. AG, a typical MADS-box transcription factor, was reported to play a pivotal role in promoting the determinate development of the floral meristem by limiting stem cell proliferation (Yanofsky *et al.* 1990, Sablowski 2007). *AsAGAMOUS*, a homolog of *AtAGAMOUS*, was highly expressed during

initial steps of flower development when the reproductive organs were specified. This finding is in agreement with reported conserved expression patterns of *AG* orthologs in other species (Kim *et al*. 2005, Theissen and Melzer 2007). Notably, *AsAGAMOUS* was highly expressed in the stamen and pistil, suggesting a putative role in pollination and reproduction.

In *Arabidopsis*, *AGL15* was identified as a putative regulator of floral organ abscission, and its expression is largely induced during the floral organ abscission (Patharkar *et al.* 2015). We also analyzed the expression of *AsAGL15* during different stages of flowering (including three abscission stages: IV, V, and VI). The expression of *AsAGL15* was much higher during stages V/VI than during stage IV, indicating involvement of *AsAGL15* in floral organ abscission. Some members of the AGL12 MADS-box subgroup have also been reported to regulate flowering transition and flowering-time control in various plant species. *OsMADS26*, a rice AGL12 group MADS-box gene, shows a similar function to *AtAGL12* in flowering-time control and reproductive organ development (Lee *et al.* 2008). Interestingly, *AsAGL12* was significantly expressed in flower bud and inhibited during flower development. Further studies are needed to reveal how *AsAGL12* controls sugar apple flower development.

In *Arabidopsis*, three closely related and functionally redundant MADS-box genes, *SEPALLATA1/2/3* (*SEP1/2/3*), are required for B and C functions by contributing to 'floral state' specification, under the 'ABC' model of floral identity (Pelaz *et al.* 2000, Zahn *et al.* 2005). In our study, two homolog of the SEP subfamily were identified in sugar apple. *AsSEP1* did not change notably during development, while the expression of its homologous gene, *AsSEP3*, peaked during flower developmental stage II and decreased during subsequent stages, suggesting that *AsSEP3* rather than *AsSEP1* may play an important role in flower bud formation in this species (Fig. 5). Furthermore, these two SEP subfamily genes show similar expression patterns in different floral organs. In sugar apple, *AsSEP1* and *AsSEP3* showed the highest expression in the pistil and lowest expression in the sepal. During flower development, organ-specific expression patterns of different MADS-box genes, including SEP genes, coordinate to control the developmental fates of floral organs (Theissen and Saedler 2001, Causier *et al.* 2010).

In previous reports, MADS-box genes were shown to be affected by application of phytohormones such as GA (Moon *et al*. 2003), SA (Martínez *et al*. 2004), and ABA (Puig *et al*. 2013). GA functions not only to promote the growth of plant organs, but also to induce certain developmental switches or phase changes. In *Arabidopsis*, physiological and genetic experiments have implicated specificity of GA in the autonomous pathway of flowering. Exogenous application of GA accelerates flowering in wild-type *Arabidopsis* (Yu *et al*. 2012). The role of GAs in flowering in perennial plants particularly in fruit trees has been studied (Remay *et al*. 2009). The expression of *AsAGL12* was inhibited by GA treatment, indicating an involvement of *AsAGL12* in GA-mediated flowering regulation. Application of ABA was reported to promote flowering in fruit trees such as litchi (Cui *et al.* 2013). Meanwhile, *AsAGL12* expression was induced by ABA treatment at 2 h and 4 h. AsAGL12 may participate in flowering by regulating the ABA-signalling pathway. Similarly, *AsAGAMOUS* was down-regulated by GA treatment and up-regulated by ABA. These results suggest that *AsAGL12* and *AsAGAMOUS* may be involved in the pathways of GA- and ABA-regulated flowering. Under stress conditions, exogenous SA application promotes the flowering response by up-regulation of *PnFT2* expression, which in turn induces flowering in *Pharbitis nil* (Yamada and Takeno 2014). The expression of *AsSEP1* and *AsAGL15* was induced by SA treatment; however, the mechanism behind SA signalling and flowering in sugar apple remains unclear.

Conclusions

Despite the importance of MADS-box genes in plant development, few studies have focused on these genes in sugar apple. In this study, seven sugar maple MADS-box genes were identified and analyzed. A phylogenetic comparison of sugar apple and *Arabidopsis* MADS-box genes clustered the seven sugar apple MADS-box genes into six different clades, including the MIKC*, AG, AGL12, AGL15, SEP, and Mγ clades. Expressions of sugar apple MADS-box genes in different tissues and floral organs suggest diverse functions for members of this gene family. In addition, the seven MADS-box genes were regulated by various phytohormones: GA, ABA, and SA. The genetic, physiological, and mechanistic findings from this study will facilitate further research on MADS-box genes in sugar apple and other woody plants.

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