

Article

Genome-Wide Identification and Characterization of MADS-box Family Genes Related to Floral Organ Development and Stress Resistance in *Hevea brasiliensis* Müll. Arg.

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Abstract: Elucidating the genetic mechanisms associated with the transition from the vegetative to reproductive phase in the rubber tree has great importance for both theoretical guidance and practical application to yield genetic improvement. At present, many transcription factors, including those that belong to the MADS-box gene family, have been revealed to have roles in regulating the transition from vegetative growth to reproductive growth. However, to the best of our knowledge, the Mad-box gene family from *H. brasiliensis* Müll. Arg. has not been characterized in detail. To investigate members of the HbMADS-box gene family associated with floral organ and inflorescence development in *H. brasiliensis*, we performed genome-wide identification and analysis of the MADS-box gene family related to flower development in *H. brasiliensis*, and a total of 20 MADS-box genes were newly identified in the *H. brasiliensis* genome. Expression profiling revealed that HbMad-box genes were differentially expressed in various tissues, which indicated that HbMad-box genes may exert different functions throughout the life cycle. Additionally, 12 genes (HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbCMB1-L, HbAGL6, HbAGL8, HbAP1, HbAG, HbDEFL, HbTT16, and HbPADS2) were found to be associated with the differentiation of flower buds and may be involved in flower development in *H. brasiliensis*. All of these floral-enriched HbMADS-box genes were regulated by hormone, salt, cold, high-temperature, and drought stresses. The present study is the first to carry out the genome-wide identification and analysis of the MADS-box gene family related to flower development in *H. brasiliensis*, and 20 new HbMad-box genes were identified in *H. brasiliensis*. Most of the newly identified HbMad-box genes were found to be associated with the differentiation of flower buds and may be involved in flower development in *H. brasiliensis*. Our results demonstrated that HbMad-box genes may be multifunctional regulators that have roles in distinct aspects of development, and are mainly involved in the maintenance of floral organ and inflorescence development.

Keywords: *Hevea brasiliensis* Müll. Arg.; HbMad-box genes; conserved domains; gene structures; expression profiles; stress treatments

1. Introduction

Natural rubber (NR) is an important industrial and strategic raw material, and has been applied to many aspects of social production [1]. Although more than 2000 plant species in the world are

considered to be latex producers, the rubber tree (*Hevea brasiliensis* Müll. Arg.) is the only commercial source of NR because of its high yield and the excellent physical properties of its rubber products [2], and it supplied 92% of the 10.2 million tons of NR consumed worldwide in 2016 [3].

With the rapid development of the world economy, the consumption of NR in major industrial countries is increasing year by year. To meet the growing demand for NR, it is necessary to expand the planting area of rubber trees. However, the rubber tree originates from the Amazon rainforest, so the planting area must be located in sub-tropical to tropical zones [4]. At present, 95% of rubber trees in the world are now mainly cultivated in South-East Asia, so the regions suitable for planting rubber trees are very limited. Therefore, there is an urgent need to improve the rubber yield per area.

To the best of our knowledge, breeding new varieties is one of the most effective approaches to increasing rubber yield per hectare. However, breeding experiments to yield genetic improvement of rubber trees are very inefficient and time-consuming, mainly because of the rubber tree's long life cycle of more than 30–35 years; it is immature for five to eight years until the rubber tree reaches the age of commercial productivity [5,6], and takes more than three decades to breed and select new clones for commercial production [7]. Furthermore, due to the low rate of success for controlled pollinations, genetic improvements of *H. brasiliensis* are very difficult and slow [8]. Therefore, research on the genetic mechanisms that affect the transition from the vegetative to reproductive phase in *H. brasiliensis* can provide insight for producing advantageous genetic improvement methods for controlling reproduction by genetic engineering and accelerate *Hevea* breeding.

In flowering plants, the transition from vegetative growth to reproductive growth is an important developmental process that involves many gene regulatory processes [9]. To date, many researchers have attempted to elucidate the functional genes in association with the transition from the vegetative growth to reproductive growth of plants. It is worth noting that many transcription factors (TFs), including those that belong to the MADS-box gene family, have been demonstrated to have roles in regulating the transition from vegetative growth to reproductive growth [10]. However, to the best of our knowledge, the rubber tree MADS-box gene family has not been characterized in detail.

As a floral homeotic gene family, the MADS-box gene family was previously identified and investigated in the model plants *Arabidopsis* Heynh. in Holl & Heynh. and *Nicotiana tabacum* L. [11–13], and has evolutionarily conserved DNA-binding domains, called the MADS-box [14]. Typically, the MADS-box protein sequences can be divided into four characteristic domains from the N to the C terminus: the MADS-box (M), intervening (I), keratin-like (K), and C-terminal (C) domains [15]. In plants, based on the structural features, MADS-box TFs usually contain two main groups—type I (M-type) and type II (MIKC-type) genes [16]; whereas, the type II genes can be categorized into MIKCc- and MIKC*-type [17]. In previous studies, it has been reported that this superfamily encodes transcriptional regulators that are involved in various processes, including floral organ development [18,19], root development [20], leaf development [21], fruit development and maturation [22–24], and embryonic development [25,26]. In addition to growth and development-related functions, some MADS-box genes also play important roles in response to stress stimuli [27,28]. For instance, MADS-box genes have already been proved to play important roles under low temperature stress in tomato plants [29], while several MADS-box genes have been demonstrated to take part in cold, salt, and drought responses in rice [30]. Furthermore, a few MADS-box genes have been shown to be affected by the application of hormones and they exhibited differential expression in response to cytokinin, gibberellin [31], ethylene [32], and auxin [33] application in other plants.

Despite the fact that the MADS-box gene plays a great role in plant growth and development, only a few MADS-box genes have been identified and characterized in the rubber tree to date [34]. For example, previous studies have found that HbAGL62 is a specific expression in flowers and embryos, and highly expressed in the flower bud differentiation stage, which indicated that HbAGL62 might play an important role in flowering regulation of the rubber tree [35]; MADS27 is highly expressed in the flower buds of rubber trees and may participate in the flowering process of rubber trees [36]; HbMADS1 and HbMADS3 have highly frequent transcriptions in the laticifer

cells and somatic embryogenesis, and their transcriptions are induced in the laticifer cells by jamic acid, which indicates that HbMADS1 and HbMADS3 may be important in natural rubber biosynthesis and somatic embryogenesis in the rubber tree [37]. In the present research, we newly identified 20 MADS-box genes in the rubber tree, and analyzed their gene structure and phylogenetic relationships. To identify differentially expressed patterns of Mad-box genes in various tissues, the 20 MADS-box genes of *H. brasiliensis* were detected and analyzed using real-time quantitative PCR (RT-qPCR). Furthermore, to understand the responses of HbMADS-box genes to various stresses, the expression profiles of 12 floral organ-specific HbMADS-box genes were examined in leaves of *Hevea* seedlings after hormone, salt, cold, high-temperature, and drought stress treatments.

2. Materials and Methods

2.1. Plant Materials and Treatments

12-year-old rubber tree clones CATAS 7-33-97 were used as the experimental material in this study. The rubber trees were grown under normal field conditions at the Experimental Station of the Rubber Research Institute, the Chinese Academy of Tropical Agricultural Sciences (Danzhou, Hainan, China). The fresh tissues and organs (including roots, stem, stem tips, leaves, labeled bark, xylem, latex, fruits, inflorescence, male flowers, and female flowers) were collected from 12-year-old mature trees of CATAS 7-33-97 during the flowering period. Each sample was harvested on average from five trees, and three biological replicates were taken from each sample (the image of some samples shown in Figure S1). Then, the prepared samples were frozen in liquid nitrogen and transferred to a -80°C refrigerator for RNA separation.

The tissue culture seedlings of CATAS 7-33-97 were treated with cold, high temperature, and drought stress. Each treatment was set up with three replicates, and each replicate consisted of five seedlings. Under the cold stress condition, the seedlings were grown in a culture incubator set at 5°C and continuous illumination. For high-temperature stress treatment, the tissue-cultured seedlings were planted at 40°C and maintained at a relative humidity of 80% in the incubator. Leaf samples of 0, 3, 6, 12, and 24 h treated with low and high temperature stresses were collected for RNA extraction. For drought stress treatments, the tissue-cultured plants were grown in Hoagland nutrient solutions [38] containing 20% PEG6000, and incubated at different times (0 h, 3 h, 6 h, 12 h, 1 day, 3 day, 4 day, and 7 day). Then, the leaf samples of each drought-treated time point were collected for RNA extraction, and samples from untreated plants were used as controls.

For hormone and salt treatments, the tissue-cultured seedlings were treated with abscisic acid (ABA) ($200\text{ }\mu\text{mol/L}$), gibberellin (GA) ($100\text{ }\mu\text{mol/L}$), and high salt (1 M NaCl), respectively. Among these chemicals, ABA and GA were diluted in distilled water that contained 0.05% (*v/v*) ethanol. The diluted ABA, GA, and NaCl solutions were sprayed on the leaves and stems of seedlings until the runoff occurred. For control plants, the distilled water that contained 0.05% (*v/v*) ethanol was sprayed on the leaves and stems of seedlings. Leaf samples were harvested 0, 0.5, 2, 6, 12, 24, and 48 h after treatments. In all treatments, one leaf from each of the five plants was taken and mixed together for RNA extraction.

2.2. RNA Isolation and First-Strand cDNA Synthesis

Total RNA was isolated from the collected samples by the described methods [39], and the extracted RNA was digested with DNase (Promega, Madison, WI, USA) to remove genomic DNA contamination. The integrity and concentration of the RNA samples was detected by 1.5% agarose gel electrophoresis and NanoDrop 2000 (Thermo Scientific Inc., Waltham, MA, USA), respectively. Then, the RNA samples were reverse transcribed into First-strand cDNA with the RevertAid™ First Strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan).

2.3. Identification and Isolation of Mad-box Genes in *H. brasiliensis*

Twenty full-length cDNA sequences of *H. brasiliensis* Mad-box genes were obtained from RNA sequencing. The cDNA sequences of these genes were compared with the Transcriptome Shotgun Assembly (TSA) and Expressed Sequence Tags (EST) of *H. brasiliensis* in the NCBI database (<http://www.ncbi.nlm.nih.gov/>), or searched against the *Hevea* genome database. Then, the NCBI ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and Softberry (<http://linux1.softberry.com/>) were used to determine open reading frames (ORFs) of candidate mRNA or genome DNA sequences. In addition, in order to confirm the presence of the Mad-box domain, all the candidate HbMad-box genes were further validated by conserved domain searching using CDD (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and InterProScan (<http://www.ebi.ac.uk/interpro/scan.html>). After the similarity comparison, ORF Finder, and conserved domain searching, redundant sequences were removed and the sequences of the HbMad-box gene were obtained.

The gene-specific primers used to amplify the corresponding full-length cDNA sequences of HbMad-box genes were designed by Primer 3.0 (<http://primer3.ut.ee/>). The primer pairs for all HbMad-box genes are listed in Table S1. RT-PCR amplification of HbMad-box genes was conducted using Pyrobest™ DNA polymerase (TaKaRa, Japan) according to the instructions.

2.4. Protein Properties and Gene Structure Analysis of HbMad-box Genes

We used the ProtParam tool (<http://web.expasy.org/protparam/>) to predict the theoretical molecular weight (Mw) and isoelectric point (PI) of HbMad-box gene proteins. To further analyze the structural diversity of HbMADS-box genes, the exon–intron structures of HbMad-box genes were identified by comparing the coding sequence with their corresponding genomic sequence using the FGENESH-C tool (<http://linux1.softberry.com>), as previously described [40].

2.5. Multiple Sequence Alignments and Phylogenetic Analysis

In the present study, amino acid sequence identities and multiple alignments of 26 HbMad-box proteins were calculated using DNAMAN6.0. To examine the evolutionary history and phylogenetic relationships of the HbMADS-box genes, we constructed a phylogenetic tree using MEGA6.0 (<http://www.megasoftware.net/>) based on multiple sequence alignment of HbMADS-box TFs from *Arabidopsis*, *Oryza sativa* L., *Vitis vinifera* L., *Jatropha carcas* L., and *H. brasiliensis*.

2.6. Quantitative Real-Time PCR (qRT-PCR) Analysis

The real-time quantitative RT-PCR (qRT-PCR) was performed according to the following procedures: 94 °C for 30 s, 94 °C for 5 s, followed by 40 cycles, 60 °C for 15 s, and 72 °C for 10 s. The reaction volume was 20 µL: including 60 ng cDNA per sample, 1×SYBR® Premix Ex Taq™ (TaKaRa, Shiga, Japan), and 0.4 µM per primer. The reaction was carried out in 96-well plates using the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). The 18S rRNA gene (GenBank accession No.: AB268099) was used as a reference gene in the qRT-PCR reaction [41]. After the reaction, we used Bio-Rad CFX Manager Software 3.0 (Bio-Rad, Hercules, CA, USA) to analyze and visualize the data as previously described [42]. All primers used for qRT-PCR analysis were designed by Primer3.0 (<http://frodo.wi.mit.edu/primer3>). The primer sequences are given in Table S2.

2.7. Statistical Analysis

Data and graphical analysis was performed with Sigma Plot 12 software (Systat Software Inc., San Jose, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of all HbMad-box genes [43]. The data are represented by the mean ± SD (standard deviation) of the three biological repeats. The statistical significance of the values was determined by a *t*-test.

3. Results

3.1. Subsection Identification and Characterization of HbMad-box Genes in *H. brasiliensis*

The 76 candidate HbMADS-box genes were checked by CDS and Inter-ProScan to confirm the existence of the MADS-box domain. After deleting the redundant sequences, a total of 26 non-redundant HbMADS-box genes (designated as HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbCMB1-L, HbAGL6, HbAGL8, HbAP1, HbAGL12, HbAG, HbAGL11, HbAGL15, HbSVP1, HbSVP2, HbTT16, HbDEFL, HbPMADS2, HbAGL30, HbAGL61, HbAGL65, HbMADS1, HbMADS2, HbMADS3, HbMADS4, HbMADS5, and HbMADS27) with complete open reading frames (ORFs) were identified in the rubber tree (Table 1), which included six previously reported HbMADS-box TFs (HbMADS1, HbMADS2, HbMADS3, HbMADS4, HbMADS5, and HbMADS27).

Table 1. Characteristics of HbMADS-box genes family in *Hevea brasiliensis* Müll. Arg.

Gene Name	GenBank Accession No.	ORF Length (bp)	Protein		
			Length (aa)	Mw (kDa)	PI
HbSEP	KY471153	732	243	28.00	8.88
HbAGL9.1	KY471149	732	243	27.77	8.21
HbAGL9.2	KY471165	723	240	27.40	8.8
HbCMB1	KY471159	729	242	27.57	8.36
HbCMB1-L	KY471157	729	242	27.87	7.6
HbAGL6	KY471160	729	242	27.63	8.73
HbAGL8	KY471148	759	252	29.16	7.72
HbAP1	KY471161	726	241	28.16	8.19
HbMADS3	GU142915	720	239	27.61	9.16
HbAGL12	KY471152	609	202	23.09	6.92
HbAG	KY471158	726	241	27.78	9.08
HbAGL11	KY471166	669	222	25.52	9.34
HbMADS2	GU142914	654	217	24.93	9.23
HbAGL15	KY471151	768	255	29.27	6.68
HbMADS27	KY486278	717	238	27.49	9.25
HbSVP1	KY471154	633	210	23.69	6.86
HbSVP2	KY471156	702	234	26.60	6.06
HbMADS4	KY486276	633	210	23.67	6.36
HbTT16	KY471155	705	234	27.46	7.69
HbDEFL	KY471162	675	224	26.23	8.43
HbPMADS2	KY471147	639	212	25.12	8.4
HbAGL30	KY471163	1116	371	41.86	7.14
HbAGL61	KY471164	642	213	24.24	8.32
HbAGL65	KY471150	741	246	28.41	8.68
HbMADS5	KY486277	1077	358	40.74	6.38
HbMADS1	GU142913	738	245	27.78	8.34

ORF, open reading frame; bp, base pair; aa, amino acids; Mw, molecular weight; PI, isoelectric point.

To confirm the putative HbMADS-box genes, the complete ORF sequences of HbMADS-box genes were isolated through RNA-sequencing and PCR-based approaches. Accurate sequences of HbMADS-box genes were submitted to GenBank, and the accession numbers of HbMADS-box genes are listed in Table 1. The ORF lengths of HbMADS-box genes ranged from 609 bp (HbAGL12) to 1116 bp (HbAGL30), and encoded polypeptides that ranged from 202 to 371 amino acids (Table 1). The corresponding Mw ranged from 23.09 to 41.86 kDa, and the predicted PI varied from 6.06 (HbSVP2) to 9.34 (HbAGL11). The distribution of PI was similar to that of AtMADS-box genes; however, the length and Mw of the HbMADS-box TFs were slightly lower than those of AtMADS-box genes (Table S3).

Pairwise sequence comparisons were performed to check the sequence identities between HbMADS-box proteins. The results showed that the identities between two HbMADS-box genes ranged from 13.67% to 98.57% (Table S4). The average sequence homology between two HbMADS-box genes was 39.99%. The largest sequence identity was observed between HbMADS4 and HbSVP1 (98.5%). HbAGL30 and HbMADS3 showed the least sequence identity (13.67%).

3.2. Analysis of Conserved Domains and Structural Features of HbMad-box Proteins

Analysis of conserved domains showed that all of the deduced HbMADS-box proteins contained a highly conserved MADS-box domain of approximately 50 amino acid residues in length, a semi-conservative K domain with an obvious coiled-coil region, a less conservative I domain, and a non-conservative C domain (Figure 1), which indicates that they belonged to the MADS-box TF family. We further analyzed the conserved domain of HbMADS-box proteins, and the results indicated that for amino acids 3 (R), 17 (R), 20 (T), 23–24 (KR), 27 (G), 30–31 (KK), 34 (E), 38–39 (LC), 48 (F), and 52 (G), up to 13 sites were conserved in the MADS-box domains (Figure 1), indicating that the deduced amino acid sequences of HbMADS-box proteins shared significant homology with each other.

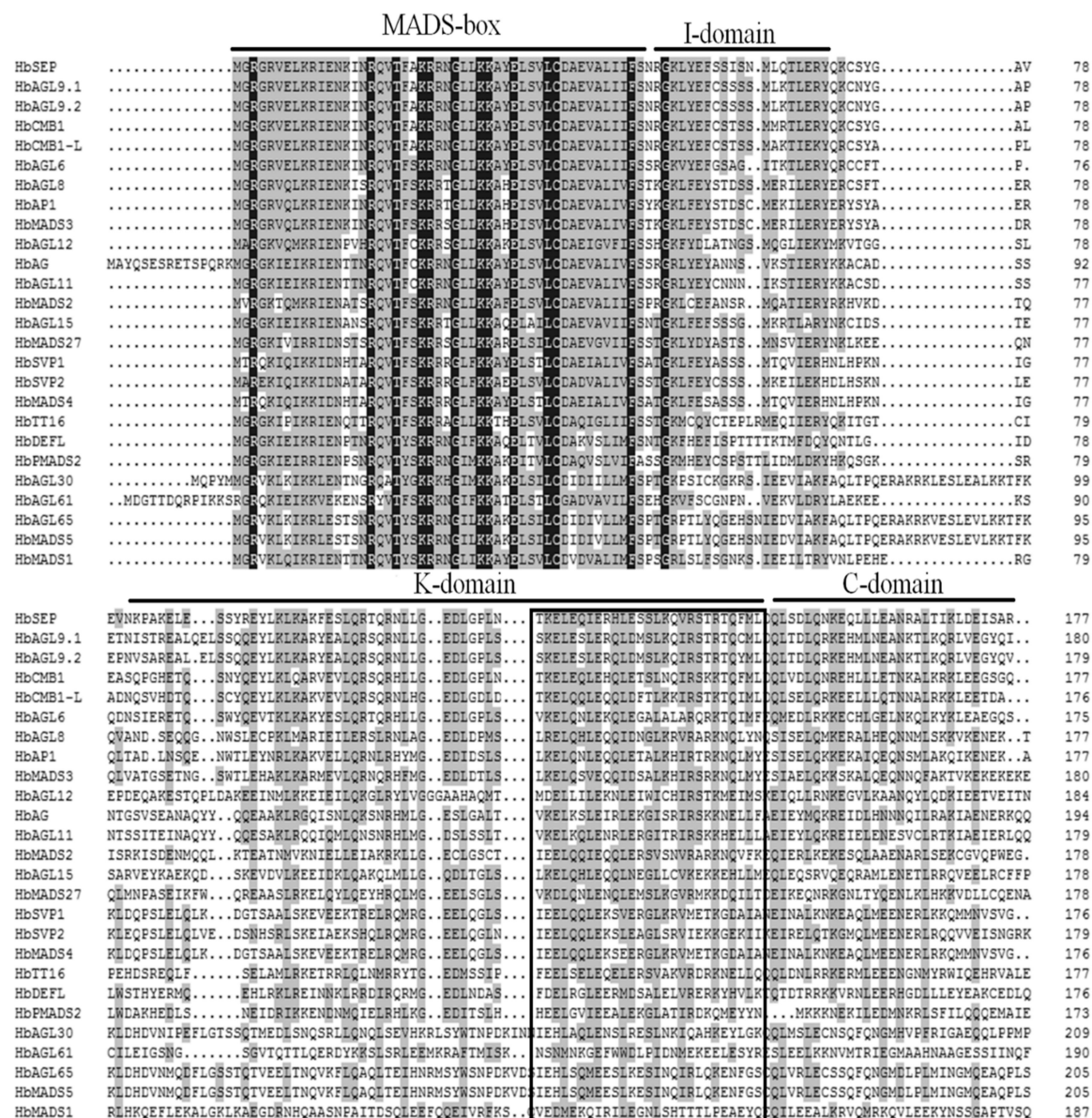


Figure 1. Phylogenetic analysis of HbMADS-box genes related proteins. MADS-box, I domain, K domain, and C domain are marked, respectively; coiled-coil domain is boxed.

The conserved motifs and structural characteristics analysis showed that all HbMADS-box genes included four conserved motifs: motifs 1 and 3 belonged to the MADS-box domains, whereas motifs 2 and 4 belonged to the K-box domains. Most HbMADS-box proteins (except HbAGL30 and HbAGL61)

contained the MADS-box and K-box domains (Figure S2), which is similar to the previously reported MADS-box conserved motifs in other species [44].

3.3. Gene Structures and Sequence Characteristics of HbMad-box Genes

Sequence analysis revealed that all of the deduced *H. brasiliensis* MADS-box genes belong to the type II (MIKC-type) MADS-box genes. These HbMad-box genes usually contained multiple introns and exons, with a maximum of 11 exons (Figure S3). According to their predicted structures, the 26 candidate HbMADS-box genes could be divided into eight groups (Table S5). The first group of HbMADS-box genes consisted of eight exons interrupted by seven introns, including eight type II HbMADS-box genes (HbSEP, HbAGL9.2, HbCMB1, HbCMB1-L, HbSVP1, HbMADS4, HbAGL65, and HbMADS5). The second and third groups included five type II HbMADS-box genes, which contained six exons interrupted by five introns (HbAGL6, HbMADS2, HbMADS27, HbSVP2, and HbTT16), and seven exons interrupted by six introns (HbAP1, HbMADS3, HbAGL12, HbAG, and HbDEFL), respectively. The fourth and fifth groups consisted of two type II HbMADS-box genes, which contained 10 exons interrupted by nine introns (HbAGL9.1 and HbAGL8), and five exons interrupted by four introns (HbAGL11 and HbPMADS2), respectively. The sixth group included two type II HbMADS-box genes (HbAGL30 and HbMADS1), which contained 11 exons interrupted by 10 introns. The seventh and eighth groups each contained one type II HbMADS-box gene (HbAGL15 and HbAGL61), which consisted of four exons interrupted by three introns, and only one exon, respectively. These results revealed that most of the HbMADS-box genes (69.2% belong to the first, second, and third groups) within the same group shared conserved exon–intron structures, and the other HbMADS-box genes (30.8% of the fourth to eighth groups) with the different exon–intron structures may belong to distinct types of HbMADS-box genes in the rubber tree (Table S5). These characteristics are consistent with the features of MADS-box genes in other flowering plants, such as *Arabidopsis*, *Oryza sativa*, and *Vitis vinifera* [45–47].

3.4. Phylogenetic Analysis of HbMad-box TFs

Based on the phylogenetic tree analysis, the 26 HbMADS-box genes could be divided into three clades (I, II, and III), which contained 11 main branches: SEP, AGL6, FUL/AP1, AGL12, AG, SOC1, AGL15, AGL17, SVP, BS, and AP3/PI subfamilies (Figure 2). Clade I contained 12 type II HbMADS-box genes, and it could be further categorized into two subclades, which contained nine and three members. Clade II contained six type II HbMADS-box genes, and it could also be divided into two subclades, and one subclade contained one member, whereas the other contained five members. Clade III contained eight type II MADS-box genes, which had two subclades, one of which contained three members and the other contained five members.

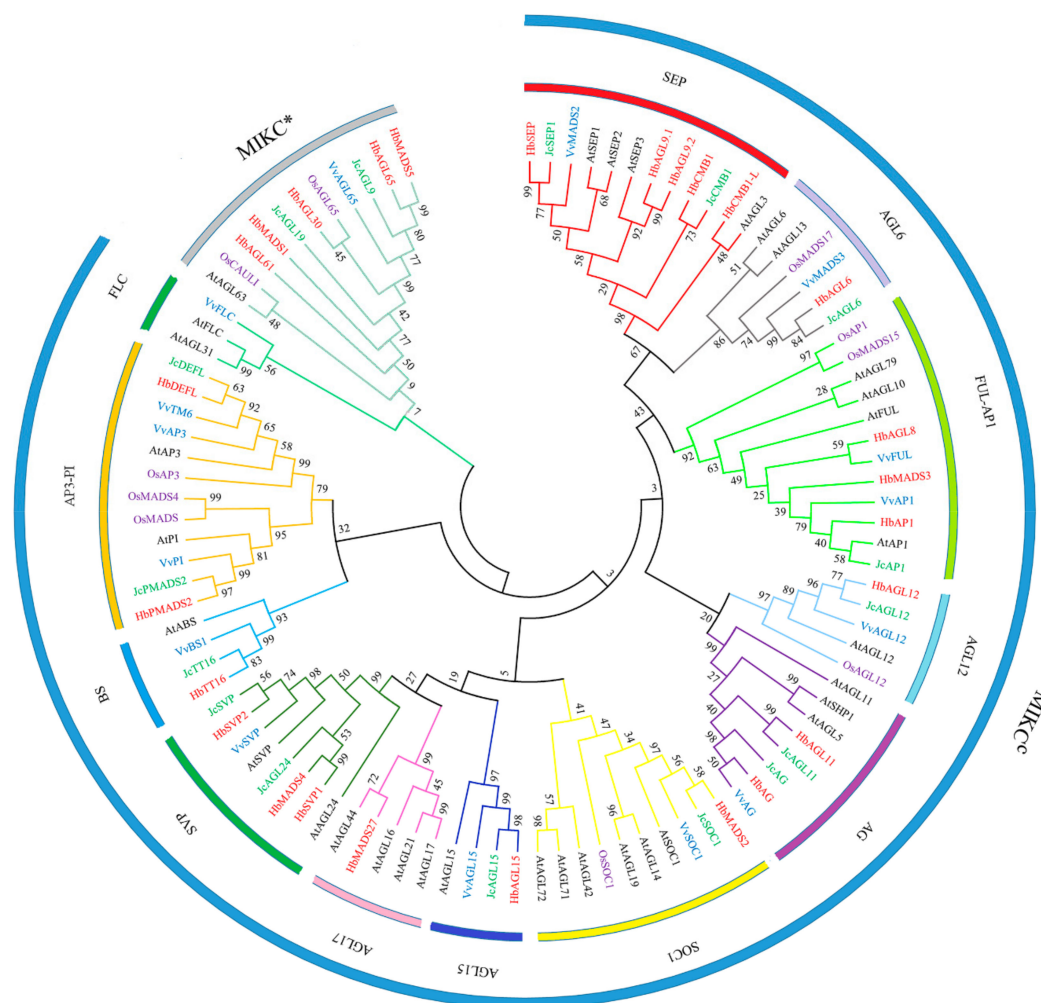


Figure 2. Phylogenetic analysis of HbMADS-box genes with other MADS-box genes by MEGA version 6.0. A phylogenetic tree of MADS-box genes was generated by the neighbor-joining (NJ) method. The multiple sequence alignment and construction of phylogenetic tree were performed with MEGA6.06 using the neighbor joining method with 1000 bootstrap replicates. The proteins were clustered and divided into three distinct sub-families. The three sub-families were further divided into 12 sub-groups.

3.5. Expression Profiles of HbMad-box Genes in Various Developmental Stages during Rubber Tree Reproductive Development

In the present study, analyzing the expression patterns of HbMADS-box genes in different tissues and organs (including roots, stem, stem tips, leaves, labeled bark, xylem, latex, fruits, inflorescence, male flowers, and female flowers) of *H. brasiliensis* by qRT-PCR, the results showed that most of the deduced HbMADS-box genes might be primarily involved in floral organ differentiation and inflorescence development. For example, HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbCMB1-L, HbAGL6, HbAG, HbDEFL, and HbPADS2 had significantly higher expression in stem tips and floral organs than other tissues; HbAP1 showed the highest expression pattern in stem tips compared to other tissues (Figure 3); HbAGL8 was significantly higher expressed in stem tips and leaves than other tissues (Figure 3); the MADS-box genes HbAGL15, HbTT16, HbMADS2, and HbAG were higher expressed in fruits than other tissues (Figure 3); and the MADS-box genes HbAGL9.2 and HbAGL6 were specifically expressed in floral organs and they were higher expressed in male flowers than other tissues (Figure 3). The higher expression of the HbMad-box genes in floral organs and shoot tips suggests that they may play a specific role in the corresponding tissues. However, in contrast, some MADS-box genes displayed different tissue expression patterns, such as HbAGL15, HbAGL30,

HbAGL61, and HbAGL65, which were almost constitutively expressed in all tested tissues (Figure 3). In addition, we also found that some HbMADS-box genes showed higher expression patterns in other specific tissues; for example, HbMADS1 and HbMADS3 were higher expressed in latex than other tissues (Figure 3), which was consistent with the findings of a previous report that found that HbMADS1 and HbMADS3 were highly expressed in laticifer cells.

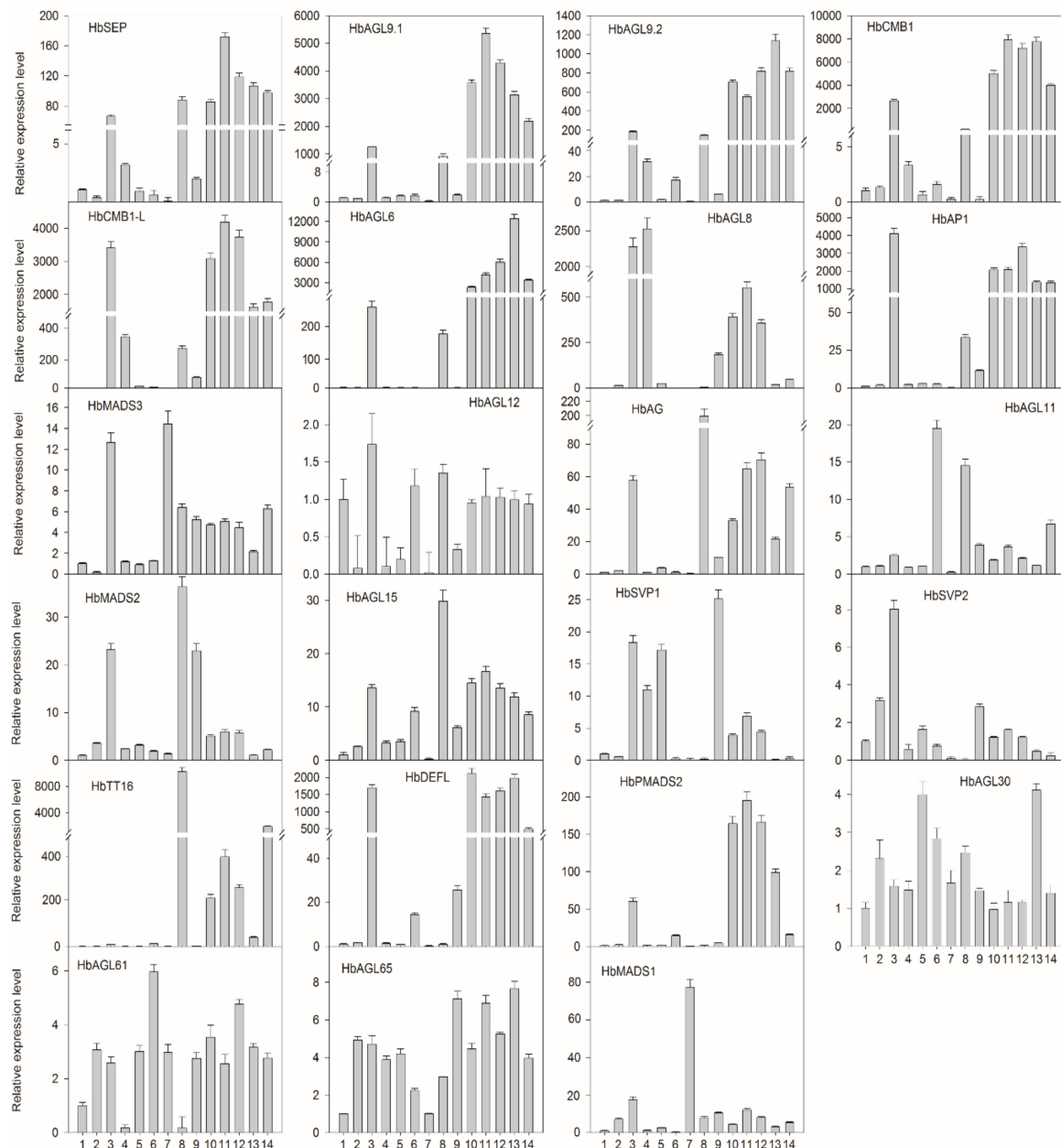


Figure 3. Relative expression levels of HbMad-box genes were determined by qRT-PCR and normalized by 18S rRNA gene expression. Relative expression levels of HbMad-box genes were determined by qRT-PCR and normalized by the 18S rRNA gene expression. For each gene, the transcript level in the root was used to normalize the transcript levels in other tissues. Values are means \pm SD (standard deviation) of three biological replicates. 1–14 represent roots; stem; stem tips; leaves; labeled bark; xylem; latex; fruits; 3 cm inflorescence; 6 cm inflorescence; 9 cm inflorescence; 12 cm inflorescence; male flowers; and female flowers, respectively.

3.6. Expression Patterns of HbMADS-box Genes in Response to Abiotic Stress

Analyzing the expression patterns of 12 floral-enriched HbMADS-box genes under cold, high-temperature, drought, and salt stresses by qRT-PCR, the results indicated that the expressions of 12 HbMADS-box genes specifically related to the floral organ were affected by low-temperature stress (Figure S4). Among the 12 cold-regulated HbMADS-box genes related to floral organ development, most of them (HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbCMB1-L, HbAGL6, HbAG, HbDEFL, and HbMADS2) were rapidly induced by cold stress, and reached the highest levels at 3 or 6 h after treatment, and then declined at 12 or 24 h after treatment. Only three HbMADS-box genes, HbAGL8, HbAP1, and HbTT16, exhibited down-regulated expression at all analyzed time-points in response to cold stress.

Under high-temperature stress, only HbDEFL expression did not significantly change (Figure S5). Of the 11 high temperature-responsive HbMADS-box genes, two genes (HbSEP and HbCMB1) were significantly up-regulated at all treated time points. Seven genes (HbAGL9.1, HbAGL9.2, HbCMB1-L, HbAGL6, HbAP1, HbAG, and HbTT16) were significantly up-regulated at at least one time point (Figure S5). By contrast, HbAGL8 and HbPADS2 were significantly down-regulated at all analyzed time points.

Under polyethylene glycol induced drought stress, the expression patterns of 12 HbMADS-box genes related to floral organ development were markedly affected (Figure S6). Of the 12 drought-responsive HbMADS-box genes, 10 genes (HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbCMB1-L, HbAP1, HbAG, HbTT16, HbDEFL, and HbMADS2) were significantly up-regulated during at least one time point after drought stress (Figure S6). Among them, HbAGL9.1, HbCMB1-L, HbAG, HbTT16, and HbDEFL were strongly affected by drought stress, reaching the highest levels that were 2-, 2.5-, 3.7-, 4-, and 5.8-fold increases compared with the control at 6 h, 12 h, 24 h, 7 day, and 4 day after treatment, respectively. Interestingly, the expression of HbAGL6 and HbAGL8 increased continuously after drought stress, and resulted in a 120- and 11-fold increase, respectively, at 7 day after treatment.

Under salt stress, the expressions of 12 floral-enriched HbMADS-box genes were regulated by salt stress. Eight genes (HbAGL9.1, HbAGL9.2, HbAGL6, HbAGL8, HbAP1, HbAG, HbDEFL, and HbPADS2) were significantly up-regulated at all of the treated time points (Figure S7). Their expression levels showed rapid up-regulation under salt stress, which peaked at 2, 48, 48, 12, 24, 48, 2, and 48 h of treatment, respectively. Four genes (HbSEP, HbCMB1, HbCMB1-L, and HbTT16) were significantly up-regulated at at least one time point under salt stress (Figure S7), and their transcripts reached maximum levels that were 2-, 2.9-, 12-, and 24-fold up-regulated compared with the control at 12, 2, 2, and 2 h after treatment, respectively.

3.7. Expression Patterns of HbMADS-box Genes in Response to Phytohormones

After ABA treatment, HbAGL9.1, HbAGL6, HbAP1, and HbPADS2 displayed obvious up-regulation across all time points (Figure S8). Their expression levels exhibited rapid up-regulation under ABA treatment, and peaked at 48, 48, 12, and 48 h after treatment, respectively. HbAGL9.2 and HbCMB1-L showed similar expression patterns; their transcripts exhibited down-regulated expression at the 6-h time point, but they were significantly up-regulated at other time points. HbSEP, HbCMB1, HbAGL8, HbAG, and HbDEFL were down-regulated at the time points of 24, 12, 0.5, 24, and 24 h compared with the control, respectively, but they displayed obvious up-regulation at other time points. In comparison, HbTT16 showed an irregular expression pattern under ABA treatment; it was up-regulated at 48 h, but was down-regulated at 0.5, 2, 12, and 24 h.

After GA3 treatment, HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbAGL6, HbAGL8, HbAP1, HbTT16, and HbDEFL showed obvious up-regulation across all time points (Figure S9). Their expression levels exhibited rapid up-regulation under GA3 treatment, and reached a maximum level at 0.5, 12, 6, 48, 12, 48, 6, and 48 h after treatment, respectively. However, HbAG and HbPADS2 were slightly down-regulated at the 24-h time point, but their expression levels showed obvious

up-regulation at other time points. By contrast, HbCMB1-L displayed an irregular expression pattern; it was strongly up-regulated at 0.5, 2, and 48 h, but was down-regulated at 6, 12, and 24 h.

4. Discussion

Understanding the flowering regulation of *H. brasiliensis* is important for accelerating the breeding process of this species. Based on studies of *Arabidopsis* and other plants, extensive efforts have been devoted to clarifying the molecular mechanism of reproductive development in plants [48,49], and techniques such as RT-qPCR and high-throughput RNA sequencing have been used in many transcriptional-level studies to identify the genes that regulate the metabolism of plant reproductive development. However, available information about TFs related to reproductive development in *H. brasiliensis* is still limited. To date, no study has been performed to determine the expression patterns of HbMad-box genes relative to rubber tree reproductive development.

As an important gene family, Mad-box genes are widespread in the plant kingdom. The number of Mad-box genes varies considerably in genomes of different species, from 20 in *Physcomitrella patens* (Hedw.) Bruch & Schimp. to 167 in *Brassica rapa* L. [50,51]. In this study, we performed genome-wide identification and analysis of the MADS-box gene family related to flower development in *H. brasiliensis*. A total of 20 MADS-box genes were newly identified in the *H. brasiliensis* genome, which was lower than the number of MADS-box genes in the woody plant *Malus pumila* Mill. [52]. A domain search using EMBL with the corresponding *H. brasiliensis* candidate protein sequences confirmed that 26 of the sequences contained a ‘MADS’ domain. We classified all 26 putative *H. brasiliensis* MADS-box proteins into three clades, which were consistent with the classifications of MADS-box gene family members in other flowering plants [53]. Sequence alignment analysis of 26 MADS-box genes revealed that their ORFs ranged from 609 to 1116 bp, and predicted protein lengths that ranged from 202 to 371 amino acids (Table 1). Subsequent gene structures analysis showed that most of the HbMad-box genes usually contained multiple introns, with a maximum of 11 introns; the exception was HbAGL61, which did not have any introns. Interestingly, we observed that only MIKC-type (no M-type) MADS-box genes existed in *H. brasiliensis*. In contrast, the number of M-type MADS-box genes in *Arabidopsis* is more than that of the MIKC^C-type [54]. Furthermore, we found that the expansion of MIKC^C-type and MIKC*-type MADS-box genes was disproportionate, and there were more MIKC^C-type (twenty-one members) than MIKC*-type (five members) MADS-box genes presented in *H. brasiliensis*, which might be related to the fact that MIKC^C-type genes conducted as functional genes to perform more complex functions in *H. brasiliensis* flower organogenesis.

Most studies on the functional identification of MADS-box genes were conducted on model plants [55–57], which also substantially contributed to revealing the diverse functions of MADS-box genes. In order to detect the expression of HbMADS-box genes in different tissues, we analyzed the expression patterns of HbMADS-box genes in 11 sample tissues and four stages of inflorescence development by qRT-PCR. The results revealed that for close orthologs of SEP and AGL6 in the rubber tree, HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbCMB1-L, and HbAGL6 showed higher expression during stem tip and floral organ development; for close orthologs of FUL-AP1 and AG in the rubber tree, HbAGL8, HbAP1, and HbAG, also exhibited higher expression during stem tip and floral organ development (Figure 3). The same expression pattern was also observed in subclade AP3-PI; HbDEFL and HbPADS2 were found to be highly expressed during stem tip and floral organ development, which indicates association with the development of reproductive organs. These findings are consistent with those of previous results that revealed the MIKC-type MADS-box gene as the flower homeotic gene that plays a dominant role in floral organ development [58].

Interestingly, HbAGL6 was progressively increased in four stages of inflorescence development, and the expression level of HbAGL6 in the male flowers was almost four-fold more than the female flowers in *H. brasiliensis*, which indicates that HbAGL6 may be involved in the development of inflorescence and floral organs, and especially participates in the development of male flowers organs (Figure 3). In addition, HbDEFL also displayed strong expression in inflorescence and

was almost four-fold more abundant in the male flowers than the female flowers in *H. brasiliensis*, which indicates that HbDEFL may play an important role in the development of male flowers. In contrast, HbTT16 showed strong expression in the fruits and female flowers, and the expression level of HbTT16 in the female flowers was almost 40-fold more than the male flowers in *H. brasiliensis*, which indicates association with the development of female organs in the rubber tree. In other plant species, TT16 homologs were also demonstrated to mediate the crosstalk between the endothelium and nucellus for the development of female organs [59,60].

Besides being involved in the essential regulation of the development of floral organs, many studies have shown that MADS-box genes also play an essential role in the response to various stresses and exhibit differential expression patterns under abiotic stress [61,62]. Rubber trees originated in the Amazon Basin of South America, but are now widely planted in the northern margin of tropical areas, such as Southeast Asia countries (e.g., Thailand, Vietnam, and southern China), which are often suffer from cold, drought, typhoon, and other abiotic stresses.

To understand the responses of HbMADS-box genes to various stresses, the expression profiles of 12 floral-enriched HbMADS-box genes were examined in leaves of *Hevea* seedlings after hormone, salt, cold, high-temperature, and drought stress treatments. In the present study, almost all of the HbMADS-box genes specifically related to floral organs exhibited changes in expression patterns when responding to hormone, salt, cold, high-temperature, and drought stresses (Figures S4–S9). Our results show that different HbMADS-box genes responded differently to abiotic stress, which indicates that stress responses of *H. brasiliensis* are regulated by many factors, involving a variety of signaling pathways. In addition, the hormone and salt stress responses of 12 floral-enriched HbMADS-box genes were more intense than those of temperature and drought stress responses. Among them, HbAGL9.2, HbAGL6, and HbAP1 were strongly induced by ABA; HbAGL9.1, HbAGL9.2, HbCMB1-L, HbAGL6, HbAGL8, HbAP1, HbTT16, and HbPADS2 were strongly induced by GA3; and HbAGL9.2, HbCMB1-L, HbAGL6, HbAGL8, HbAP1, HbTT16, and HbPADS2 were strongly induced by salt treatments, whereas their expressions were slightly affected by cold, high-temperature, and drought stresses. The expressions of HbAGL9.2, HbAGL6, and HbAP1 were strongly induced by a variety of hormones, which revealed that these genes might play a crucial role in hormone signaling in *H. brasiliensis*.

Overall, we characterized HbMADS-box genes as multifunctional regulators in *H. brasiliensis* based on tissue specific expression analysis, as well as various stress responses. Our results revealed that HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbCMB1-L, HbAGL6, HbAGL8, HbAP1, HbAG, HbDEFL, and HbPADS2 may mainly regulate the differentiation of flower buds and could help regulate reproduction in *H. brasiliensis*; whereas HbTT16 may mainly regulate the development of fruits and female organs in the rubber tree. Expression profiling revealed that different HbMADS-box genes responded differently to abiotic stresses, which indicates that abiotic stresses in *H. brasiliensis* are regulated by many factors and various signaling pathways. Our findings will promote the development of technology that can control the reproduction of *H. brasiliensis* by genetic engineering. In the near future, we will be able to verify the role of these HbMADS-box genes in the transition of vegetative to reproductive development using transgenic *H. brasiliensis*, as well as transgenic *Arabidopsis*.

5. Conclusions

In this study, 20 new HbMad-box genes were identified in the rubber tree. Subsequently, the bioinformatics characteristics, sequence identity, conserved domains, gene structure, and phylogenetic relationship of these genes were systematically analyzed. Expression profiling revealed that HbMad-box genes were differentially expressed in various tissues, which indicated that HbMad-box genes may exert different functions throughout the life cycle. Additionally, HbSEP, HbAGL9.1, HbAGL9.2, HbCMB1, HbCMB1-L, HbAGL6, HbAGL8, HbAP1, HbAG, HbDEFL, HbTT16, and HbPADS2 were found to be associated with the differentiation of flower buds and may be involved in flower development. All of these floral-enriched HbMADS-box genes were regulated by hormone, salt, cold, high-temperature, and drought stresses, which revealed that abiotic stresses in

H. brasiliensis are regulated by many factors and various signaling pathways. Our results provide a comprehensive overview of the HbMADS-box gene family related to floral development and lay the foundation for further functional characterization of this gene family in *H. brasiliensis*.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/9/6/304/s1, Figure S1: Tissue samples of rubber tree flowers, Figure S2: The conserved domain logo and motif composition of HbMADS-box genes, Figure S3: Exon-intron structures of HbMads-box genes, Figure S4: Expression profiles of the floral-enriched HbMad-box genes under cold stress, Figure S5: Expression profiles of the floral-enriched HbMad-box genes under high temperature stress, Figure S6: Expression profiles of the floral-enriched HbMad-box genes under drought stress, Figure S7: Expression profiles of the floral-enriched HbMad-box genes under salt stress, Figure S8: Expression profiles of the floral-enriched HbMad-box genes responding to ABA treatment, Figure S9: Expression profiles of the floral-enriched HbMad-box genes responding to GA3 treatment, Table S1: List of primer sequences used for HbMad-box TFs amplification, Table S2: List of primer sequences used for qRT-PCR analysis, Table S3: Sequence features of AtMADSs in *A. thaliana*. (L.) Heynh. Gene IDs, protein length, PI, and molecular weight of corresponding AtMADSs in *A. thaliana* are shown, Table S4: Percent identity matrix of HbMad-box TFs proteins, Table S5: Statistics of exon and intron number distribution among HbMad-box TFs.

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References

- Li, D.; Zeng, R.; Yan, L.; Zhao, M.; Chao, J.; Yu, L. Gene expression analysis and SNP/InDel discovery to investigate yield heterosis of two rubber tree F1 hybrids. *Sci. Rep.* **2016**, *6*, 24984. [[CrossRef](#)] [[PubMed](#)]
- Mooibroek, H.; Cornish, K. Alternative sources of natural rubber. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 355–365. [[CrossRef](#)] [[PubMed](#)]
- Ahmad, N.; Abnisaa, F.; Daud, W. Potential use of natural rubber to produce liquid fuels using hydrous pyrolysis—A review. *RSC Adv.* **2016**, *6*, 68906–68921. [[CrossRef](#)]
- Pethin, D.; Nakkanong, K.; Nualsri, C. Performance and genetic assessment of rubber tree clones in Southern Thailand. *Sci. Agric.* **2015**, *72*, 306–313. [[CrossRef](#)]
- Ahrends, A.; Hollingsworth, P.M.; Ziegler, A.D.; Fox, J.M.; Chen, H.; Su, Y. Current trends of rubber plantation expansion may threaten biodiversity and livelihoods. *Glob. Environ. Chang.* **2015**, *34*, 48–58. [[CrossRef](#)]
- Dornelas, M.C.; Rodriguez, A.P. The rubber tree (*Hevea brasiliensis* Muell. Arg.) homologue of the *LEAFY/FLORICAULA* gene is preferentially expressed in both male and female floral meristems. *J. Exp. Bot.* **2005**, *56*, 1965–1974. [[CrossRef](#)] [[PubMed](#)]
- Cubry, P.; Pujade-Renaud, V.; Garcia, D.; Espeout, S.; Le, G.V.; Granet, F. Development and characterization of a new set of 164 polymorphic EST-SSR markers for diversity and breeding studies in rubber tree (*Hevea brasiliensis* Mull. Arg.). *Plant Breed.* **2014**, *133*, 419–426. [[CrossRef](#)]
- Ó'Maoiléidigh, D.S.; Wuest, S.E.; Rae, L.; Raganelli, A.; Ryan, P.T.; Kwaśniewska, K.; Wellmer, F. Control of reproductive floral organ identity specification in *Arabidopsis* by the C function regulator AGAMOUS. *Plant Cell* **2013**, *25*, 2482–2503. [[CrossRef](#)] [[PubMed](#)]
- Yu, H.; Ito, T.; Wellmer, F.; Meyerowitz, E.M. Repression of AGAMOUS-LIKE 24 is a crucial step in promoting flower development. *Nat. Genet.* **2004**, *36*, 157–161. [[CrossRef](#)] [[PubMed](#)]
- Patharkar, O.R.; Walker, J.C. Floral organ abscission is regulated by a positive feedback loop. *PNAS* **2015**, *112*, 2906–2911. [[CrossRef](#)] [[PubMed](#)]
- Adamczyk, B.J.; Lehtishiu, M.D.; Fernandez, D.E. The MADS domain factors AGL15 and AGL18 act redundantly as repressors of the floral transition in *Arabidopsis*. *Plant J.* **2007**, *50*, 1007–1019. [[CrossRef](#)] [[PubMed](#)]
- García-Maroto, F.; Carmona, M.J.; Garrido, J.A.; Vilches-Ferrón, M.; Rodríguez-Ruiz, J.; Alonso, D.L. New roles for MADS-box genes in higher plants. *Biol. Plant.* **2003**, *46*, 321–330. [[CrossRef](#)]

13. Nam, J.; Ma, H.; Nei, M. Antiquity and evolution of the MADS-box gene family controlling flower development in plants. *Mol. Biol. Evol.* **2003**, *20*, 1435–1447. [[CrossRef](#)] [[PubMed](#)]
14. Wong, C.E.; Singh, M.B.; Bhalla, P.L. Novel members of the AGAMOUS LIKE 6 subfamily of MIKCC-type MADS-box genes in soybean. *BMC Plant Biol.* **2013**, *13*, 105. [[CrossRef](#)] [[PubMed](#)]
15. Leseberg, C.H.; Li, A.; Kang, H.; Duvall, M.; Mao, L. Genome-wide analysis of the MADS-box gene family in populus trichocarpa. *Gene* **2006**, *378*, 84–94. [[CrossRef](#)] [[PubMed](#)]
16. Kofuji, R.; Sumikawa, N.; Yamasaki, M.; Kondo, K.; Ueda, K.; Ito, M.; Hasebe, M. Evolution and divergence of the MADS-box gene family based on genome-wide expression analyses. *Mol. Biol. Evol.* **2003**, *20*, 1963–1977. [[CrossRef](#)] [[PubMed](#)]
17. Torti, S.; Fornara, F.; Vincent, C.; Andrés, F.; Nordström, K.; Göbel, U. Analysis of the *Arabidopsis* shoot meristem transcriptome during floral transition identifies distinct regulatory patterns and a leucine-rich repeat protein that promotes flowering. *Plant Cell* **2012**, *24*, 444–462. [[CrossRef](#)] [[PubMed](#)]
18. Honma, T.; Goto, K. Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* **2001**, *409*, 525–529. [[CrossRef](#)] [[PubMed](#)]
19. Lalusin, A.G.; Nishita, K.; Kim, S.H.; Ohta, M.; Fujimura, T. A new MADS-box gene (IbMADS10) from sweet potato (*Ipomoea batatas* L. Lam) is involved in the accumulation of anthocyanin. *Mol. Genet. Genom.* **2006**, *275*, 44–54. [[CrossRef](#)] [[PubMed](#)]
20. Zhang, B.; Su, X.; Zhou, X. A MADS-box gene of *Populus deltoides* expressed during flower development and in vegetative organs. *Tree Physiol.* **2008**, *28*, 929–934. [[CrossRef](#)] [[PubMed](#)]
21. Liu, J.; Xu, B.; Hu, L.; Li, M.; Su, W.; Wu, J. Involvement of a banana MADS-box transcription factor gene in ethylene-induced fruit ripening. *Plant Cell Rep.* **2009**, *28*, 103–111. [[CrossRef](#)] [[PubMed](#)]
22. Tani, E.; Polidoros, A.N.; Tsiftaris, A.S. Characterization and expression analysis of Fruitfull- and shatterproof-like genes from peach (*Prunus persica*) and their role in split-pit formation. *Tree Physiol.* **2007**, *27*, 649–659. [[CrossRef](#)] [[PubMed](#)]
23. Ge, J.; Zhao, D.; Han, C.; Wang, J.; Hao, Z.; Tao, J. Cloning and expression of floral organ development-related genes in herbaceous peony (*Paeonia lactiflora*, Pall.). *Mol. Biol. Rep.* **2014**, *41*, 6493–6530. [[CrossRef](#)] [[PubMed](#)]
24. Bemer, M.; Heijmans, K.; Airoldi, C.; Davies, B.; Angenent, G. An atlas of type I MADS box gene expression during female gametophyte and seed development in *Arabidopsis*. *Plant Physiol.* **2010**, *154*, 287–300. [[CrossRef](#)] [[PubMed](#)]
25. Thakare, D.; Tang, W.; Hill, K.; Perry, S.E. The MADS-domain transcriptional regulator AGAMOUS-LIKE15 promotes somatic embryo development in *Arabidopsis* and soybean. *Plant Physiol.* **2008**, *146*, 1663–1672. [[CrossRef](#)] [[PubMed](#)]
26. Colombo, M.; Masiero, S.; Vanzulli, S.; Lardelli, P.; Kater, M. M.; Colombo, L. AGL23, a type I MADS-box gene that controls female gametophyte and embryo development in *Arabidopsis*. *Plant J.* **2008**, *54*, 1037–1048. [[CrossRef](#)] [[PubMed](#)]
27. Lee, S.; Woo, Y.; Ryu, S.; Shin, Y.; Kim, W.; Park, K.; Lee, I.; An, G. Further characterization of a rice AGL12 group MADS-Box gene, OsMADS26. *Plant Physiol.* **2008**, *147*, 156–168. [[CrossRef](#)] [[PubMed](#)]
28. Elitzur, T.; Vrebalov, J.; Giovannoni, J.J.; Goldschmidt, E.E.; Friedman, H. The regulation of MADS-box gene expression during ripening of banana and their regulatory interaction with ethylene. *J. Exp. Bot.* **2010**, *61*, 1523–1535. [[CrossRef](#)] [[PubMed](#)]
29. Lozano, R.; Angosto, T.; Gomez, P.; Payan, C.; Capel, J.; Huijser, P. Tomato flower abnormalities induced by low temperatures are associated with changes of expression of MADS-Box genes. *Plant Physiol.* **1998**, *117*, 91–100. [[CrossRef](#)] [[PubMed](#)]
30. Arora, R.; Agarwal, P.; Ray, S.; Singh, A.K.; Singh, V.P.; Tyagi, A.K. MADS-box gene family in rice: Genome-wide identification, organization and expression profiling during reproductive development and stress. *BMC Genom.* **2007**, *8*, 242. [[CrossRef](#)] [[PubMed](#)]
31. Bonhomme, F.; Kurz, B.; Melzer, S.; Bernier, G.; Jacquemard, A. Cytokinin and gibberellin activate SaMADS A, a gene apparently involved in regulation of the floral transition in *Sinapis alba*. *Plant J.* **2000**, *24*, 103–111. [[CrossRef](#)] [[PubMed](#)]
32. Ando, S.; Sato, Y.; Kamachi, S.; Sakai, S. Isolation of a MADS-box gene (ERAF17) and correlation of its expression with the induction of formation of female flowers by ethylene in cucumber plants (*Cucumis sativus* L.). *Planta* **2001**, *213*, 943–952. [[CrossRef](#)] [[PubMed](#)]

33. Zhu, C.; Perry, S.E. Control of expression and auto regulation of AGL15, a member of the MADS-box family. *Plant J.* **2005**, *41*, 583–594. [[CrossRef](#)] [[PubMed](#)]
34. Li, H.L.; Wei, L.R.; Guo, D.; Wang, Y.; Zhu, J.H.; Chen, X.T. HbMADS4, a MADS-box transcription factor from *Hevea brasiliensis*, negatively regulates HbSRPP. *Front. Plant Sci.* **2016**, *7*, 1709–1712. [[CrossRef](#)] [[PubMed](#)]
35. Cai, H.B.; Tu, M.; Hua, Y.W.; Huang, H.S. Cloning and expression analysis of HbAGL62 of rubber tree (*Hevea brasiliensis* Arg.). *Chin. J. Trop. Crops.* **2012**, *33*, 1766–1771.
36. Wang, H.; Li, L.; Liang, Z.; Wu, Y.J. Cloning and bioinformatic analysis of the gene HbMADS-27 from *Hevea brasiliensis*. *Chin. J. Trop. Agric.* **2013**, *33*, 19–24.
37. Li, H.L.; Wang, Y.; Guo, D.; Tian, W.M.; Peng, S.Q. Three MADS-box genes of *Hevea brasiliensis* expressed during somatic embryogenesis and in the laticifer cells. *Mol. Biol. Rep.* **2011**, *38*, 4045–4052. [[CrossRef](#)] [[PubMed](#)]
38. Cheng, H.; Chen, X.; Zhu, J.S.; Huang, H.S. Overexpression of a *Hevea brasiliensis* ErbB-3 Binding protein 1 Gene Increases Drought Tolerance and Organ Size in *Arabidopsis*. *Front. Plant Sci.* **2016**, *7*, 1703. [[CrossRef](#)] [[PubMed](#)]
39. Xu, J.; Aileni, M.; Abbagani, S.; Zhang, P. A reliable and efficient method for total RNA isolation from various members of spurge family (*Euphorbiaceae*). *Phytochem. Anal.* **2010**, *21*, 395–398. [[CrossRef](#)] [[PubMed](#)]
40. Solovyev, V. Statistical Approaches in Eukaryotic Gene Prediction. In *Handbook of Statistical Genetics*; John Wiley & Sons, Ltd.: Clifton, NJ, USA, 2004; pp. 7–39.
41. Tang, C.; Huang, D.; Yang, J.; Liu, S.; Sakr, S.; Li, H. The sucrose transporter HbSUT3 plays an active role in sucrose loading to laticifer and rubber productivity in exploited trees of *Hevea brasiliensis* (para rubber tree). *Plant Cell Environ.* **2010**, *33*, 1708–1720. [[CrossRef](#)] [[PubMed](#)]
42. Long, X.; He, B.; Wang, C.; Fang, Y.; Qi, J.; Tang, C. Molecular identification and characterization of the pyruvate decarboxylase gene family associated with latex regeneration and stress response in rubber tree. *Plant Physiol. Biochem.* **2015**, *87*, 35–44. [[CrossRef](#)] [[PubMed](#)]
43. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)] [[PubMed](#)]
44. Nakatsuka, T.; Saito, M.; Yamada, E.; Fujita, K.; Yamagishi, N.; Yoshikawa, N. Isolation and characterization of the C-class MADS-box gene involved in the formation of double flowers in Japanese gentian. *BMC Plant Biol.* **2015**, *15*, 182. [[CrossRef](#)] [[PubMed](#)]
45. Liu, Y.; Zhang, D.; Ping, J.; Li, S.; Chen, Z.; Ma, J. Innovation of a regulatory mechanism modulating semi-determinate stem growth through artificial selection in soybean. *PLoS Genet.* **2016**, *12*, e1005818. [[CrossRef](#)] [[PubMed](#)]
46. Par Enicová, L.; Folter, S.D.; Kieffer, M.; Horner, D.S.; Favalli, C.; Busscher, J. Molecular and Phylogenetic Analyses of the Complete MADS-Box Transcription Factor Family in *Arabidopsis*: New openings to the MADS world. *Plant Cell* **2003**, *15*, 1538–1551. [[CrossRef](#)]
47. Díazrielme, J.; Lijavetzky, D.; Martínezzapater, J.M.; Carmona, M.J. Genome-wide analysis of MIKCC-type MADS-box genes in grapevine. *Plant Physiol.* **2009**, *149*, 354–369. [[CrossRef](#)] [[PubMed](#)]
48. Zinta, G.; Khan, A.; Abdelgawad, H.; Verma, V.; Srivastava, A.K. Unveiling the redox control of plant reproductive development during abiotic stress. *Front. Plant Sci.* **2016**, *7*, 700. [[CrossRef](#)] [[PubMed](#)]
49. Fromm, S.; Braun, H.; Peterhansel, C. Mitochondrial gamma carbonic anhydrases are required for complex I assembly and plant reproductive development. *New Phytol.* **2016**, *211*, 194–207. [[CrossRef](#)] [[PubMed](#)]
50. Yi, J.M.; Huang, T.; Huang, Y.; Chen, D.H. Phylogenetic analysis of MADS-box gene family in *Physcomitrella patens*. *Plant Physiol. J.* **2015**, *51*, 197–206. [[CrossRef](#)]
51. Saha, G.; Park, J.I.; Jung, H.J.; Ahmed, N.U. Genome-wide identification and characterization of MADS-box family genes related to organ development and stress resistance in *Brassica rapa*. *BMC Genom.* **2015**, *16*, 178. [[CrossRef](#)] [[PubMed](#)]
52. Kumar, G.; Arya, P.; Gupta, K.; Randhawa, V.; Acharya, V.; Singh, A.K. Comparative phylogenetic analysis and transcriptional profiling of MADS-box gene family identified DAM and FLC-like genes in apple (*Malus domestica*). *Sci. Rep.* **2016**, *6*, 20695. [[CrossRef](#)] [[PubMed](#)]
53. Grimplet, J.; Martínez-Zapater, J.M.; Carmona, M.J. Structural and functional annotation of the MADS-box transcription factor family in grapevine. *BMC Genom.* **2016**, *17*, 80. [[CrossRef](#)] [[PubMed](#)]
54. Nam, J.; Kim, J.; Lee, S.; An, G.; Ma, H.; Nei, M. Type I MADS-box genes have experienced faster birth-and-death evolution than type II MADS-box genes in angiosperms. *PNAS* **2004**, *101*, 1910–1915. [[CrossRef](#)] [[PubMed](#)]

55. Smaczniak, C.; Immink, R.G.; Angenent, G.C.; Kaufmann, K. Developmental and evolutionary diversity of plant MADS-domain factors: Insights from recent studies. *Development* **2012**, *139*, 3081–3098. [[CrossRef](#)] [[PubMed](#)]
56. Masiero, S.; Li, M.A.; Will, I.; Hartmann, U.; Saedler, H.; Huijser, P. Incomposita: A MADS-box gene controlling prophyll development and floral meristem identity in *antirrhinum*. *Development* **2004**, *131*, 5981–5990. [[CrossRef](#)] [[PubMed](#)]
57. Toshitsugu, N.; Junji, K.; Masaki, F.; Mamiko, K.; Nao, I.; Hideo, M. Macrocalyx and jointless interact in the transcriptional regulation of tomato fruit abscission zone development. *Plant Physiol.* **2012**, *158*, 439–450. [[CrossRef](#)]
58. Huang, Q.; Li, W.; Fan, R.; Chang, Y. New mads-box gene in fern: Cloning and expression analysis of dfmads1 from *Dryopteris fragrans*. *PLoS ONE* **2014**, *9*, e86349. [[CrossRef](#)] [[PubMed](#)]
59. Lepiniec, L.; Debeaujon, I.; Routaboul, J.M.; Baudry, A.; Pourcel, L.; Nesi, N.; Caboche, M. Genetics and biochemistry of seed flavonoids. *Annu. Rev. Plant Biol.* **2006**, *57*, 405–430. [[CrossRef](#)] [[PubMed](#)]
60. Xu, W.; Fiume, E.; Coen, O.; Pechoux, C.; Lepiniec, L.; Magnani, E. Endosperm and nucellus develop antagonistically in *Arabidopsis* seeds. *Plant Cell* **2016**, *28*, 1343–1360. [[CrossRef](#)] [[PubMed](#)]
61. Chen, G.; Deng, W.; Peng, F.; Truksa, M.; Singer, S.; Snyder, C.L. *Brassica napus* TT16 homologs with different genomic origins and expression levels encode proteins that regulate a broad range of endothelium-associated genes at the transcriptional level. *Plant J.* **2013**, *74*, 663–677. [[CrossRef](#)] [[PubMed](#)]
62. Zhang, Z.; Li, H.; Zhang, D.; Liu, Y.; Fu, J.; Shi, Y. Characterization and expression analysis of six MADS-box genes in maize (*Zea mays* L.). *J. Plant Physiol.* **2012**, *169*, 797–806. [[CrossRef](#)] [[PubMed](#)]



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