Genome-wide analysis of the MYB gene family in pumpkin

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The *MYB* gene family exerts significant influence over various biological processes and stress responses in plants. Despite this, a comprehensive analysis of this gene family in pumpkin remains absent. In this study, the *MYB* genes of *Cucurbita moschata* were identified and clustered into 33 groups (C1-33), with members of each group being highly conserved in terms of their motif composition. Furthermore, the distribution of 175 *CmoMYB* genes across all 20 chromosomes was found to be non-uniform. Examination of the promoter regions of these genes revealed the presence of cis-acting elements associated with phytohormone responses and abiotic/biotic stress. Utilizing quantitative real-time polymerase chain reaction (qRT-PCR), the expression patterns of 13 selected *CmoMYB* genes were validated, particularly in response to exogenous phytohormone exposure and various abiotic stressors, including ABA, SA, MeJA, and drought treatments. Expression analysis in different tissues showed that *CmoMYB* genes are expressed at different levels in different tissues, suggesting that they are functionally divergent in regulating growth and abiotic stresses. These results provide a basis for future studies to characterize the function of the *MYB* gene family under abiotic stresses in pumpkins.

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Abstract

The *MYB* gene family exerts significant influence over various biological processes and stress responses in plants. Despite this, a comprehensive analysis of this gene family in pumpkin remains absent. In this study, the *MYB* genes of *Cucurbita moschata* were identified and clustered into 33 groups (C1-33), with members of each group being highly conserved in terms of their motif composition. Furthermore, the distribution of 175 *CmoMYB* genes across all 20 chromosomes was found to be non-uniform. Examination of the promoter regions of these genes revealed the presence of cis-acting elements associated with phytohormone responses and abiotic/biotic stress. Utilizing quantitative real-time polymerase chain reaction (qRT-PCR), the expression patterns of 13 selected *CmoMYB* genes were validated, particularly in response to exogenous phytohormone exposure and various abiotic stressors, including ABA, SA, MeJA, and drought treatments. Expression analysis in different tissues showed that *CmoMYB* genes are expressed at different levels in different tissues, suggesting that they are functionally divergent in regulating growth and abiotic stresses. These results provide a basis for future studies to characterize the function of the *MYB* gene family under abiotic stresses in pumpkins.

Keywords: *MYB*; *cis*-acting elements; ABA; SA; MeJA; abiotic stresses

1.Introduction

Transcription factors (TFs) are characterized by the presence of distinct structural domains, including a nuclear localization signal domain, DNA-binding domain, transcription regulation domain, and oligomerization domain [1, 2]. Depending on the structure of the domain that specifically binds to DNA sequences, the TFs can be classified into many families [3]. Among these families, the MYB superfamily stands out, boasting conserved MYB DNA-binding structural domains that are ubiquitously distributed across eukaryotes [4, 5]. The identification of the *MYB* gene originated from the avian myeloblastosis virus [6], followed by the discovery of *A-MYB*, *B-MYB*, and *c-MYB*, in numerous vertebrates, showcasing their involvement in the regulation of cell differentiation, proliferation, and apoptosis [7-10]. The first functional *MYB* gene found in plants was *ZmMYBC1* that involved in the regulation of anthocyanin biosynthesis [11]. The MYB domain typically comprises one to four imperfect repeats, each forming a helix-turn-helix structure spanning 50-53 amino acids [12, 13]. Notably, these repeats feature uniformly localized tryptophan residues, which congregate within the hydrophobic core of each repeat, thereby stabilizing the

three-dimensional helix-turn-helix structure of the MYB protein's DNA binding domain. With the proliferation of genome sequencing endeavors, MYB family TFs have been extensively investigated across numerous plant species. Notable examples include *Arabidopsis thaliana* (193 *AtMYBs*) [14], *Oryza sativa* (197 *OsMYBs*) [15], *Glycine max* (252 *GmMYBs*) [2], *Gossypium hirsutum* (524 *GhMYBs*) [16], *Solanum tuberosum* (253 *StMYBs*) [17], *Pyrus bretschneideri* (129 *PbMYBs*) [18].

Previous studies have established the pivotal role of the *MYB* gene family in cell development, primary and secondary metabolism, and stress responses [5, 19, 20]. Notably, the overexpression of *SpMYB* (from *Solanum pimpinellifolium* L3708) demonstrated heightened resistance to necrotrophic pathogens and enhanced salt and drought stress tolerance in tobacco [21, 22]. Similarly, transgenic rice plants overexpressing *OsMYB2P-1* exhibited increased tolerance to inorganic phosphate starvation through the promotion of primary root elongation [23]. In *Arabidopsis*, *AtMYB60* and *AtMYB61* have been implicated in root development and stomatal aperture [24, 25], while *AtMYB44*, *AtMYB52*, and *AtMYB96* have been associated with drought stress response via ABA-mediated pathways [26-28]. Despite extant studies on the MYB gene family, a comprehensive genome-wide characterization of this family remains absent in *C. moschata.*

Pumpkins, recognized for their escalating economic importance due to bioactive compounds such as carotenoids, phenolic compounds, and flavonoids, also serve as rootstocks for other cucurbit crops, contributing to enhanced tolerance against soil-borne diseases and abiotic stresses [29, 30]. In recent years, water scarcity has become increasingly important for crops because of global climate issues. The yield and quality of pumpkin can be severely affected by drought stress, so it is important to identify candidate genes responsible for drought stress tolerance. Highlighting the crucial role of the *MYB* gene family in stress responses, this research specifically concentrates on the comprehensive genome-wide identification and expression analysis of MYB transcription factors (TFs) in pumpkins under abiotic stress conditions. The investigation encompasses the construction of phylogenetic relationships among CmoMYB proteins, along with an exploration of chromosome localization, genomic structure, and motif protein composition. Subsequently, thirteen selected MYB TFs undergo scrutiny, and their expression patterns under abiotic stress are meticulously analyzed using qRT-PCR. A notable pattern of regulation emerged, with 10 genes exhibiting up-regulation and 1 gene demonstrating down-regulation following ABA treatment at

6 hours. Additionally, 11 *CmoMYB* genes displayed significant regulation, comprising 9 up-regulated and 2 down-regulated genes, excluding *CmoMYB59* (which initiated a decrease at 6 hours) and *CmoMYB3* (which initiated an increase at 6 hours) after JA treatment. After SA treatment, all *CmoMYB* genes exhibited significant regulation at 3 hours, with 11 genes up-regulated and 2 down-regulated. This study aims to lay the foundation for understanding the regulatory mechanism of the *MYB* gene.

2. Materials & Methods

2.1. Identification and characteristics analysis of the *MYB* gene family in *C. moschata*

The protein sequence database of *C. moschata* was downloaded from the Cucurbitaceae genome database (http://cucurbitgenomics.org/, accessed on 28 March 2023). In addition, the MYB sequences of *Arabidopsis thaliana* and *Oryza sativa* were obtained from the phytozome database (https://phytozome-next.jgi.doe.gov/, accessed on 28 March 2023). The CmoMYB was identified from two plants at the whole genome level using two approaches. First, functionally known MYB protein sequences of *Arabidopsis* (AtMYB4, AT4G38620) and *Oryza sativa* (OsMYB, LOC_Os08g43550) were used as query sequences to blast the proteins of the *C. moschata*. All domains of primary candidate MYB proteins were submitted to the Pfam domain database (http://pfam.xfam.org/, accessed on 28 March 2023) and the Conserved Domains Database (CDD, http://www.ncbi.nlm.nih.gov/Structure, accessed on 28 March 2023) and the profile hidden Markov models (HMMER, https://www.ebi.ac.uk/Tools/hmmer/, accessed on 28 March 2023) to further examined. All MYB protein sequences containing MYB or MYB-like domains were selected and named based on the homology of AtMYB4, respectively.

The ID numbers, genomic positions, genome sequences, and protein sequences of CmoMYB members were downloaded from the Cucurbita genome database. The ExPASy proteomics online tool (https://web.expasy.org/protparam/, accessed on 29 March 2023) was used to analyze the physiological and biochemical characteristics of CmoMYB proteins, including number of amino acids, the relative molecular mass (Mw), coding sequence (CDS), and isoelectric point (pI), etc. The subcellular localization of ComMYB proteins was predicted by using the WoLF PSORT website (https://wolfpsort.hgc.jp/, accessed on 29 March 2023) and BUSCA website (http://busca.biocomp.unibo.it/, accessed on 29 March 2023).

2.2. Construction of phylogenetic trees of MYB proteins and duplication analysis

MYB protein phylogenetic trees were constructed and CmoMYB proteins were grouped to explore the evolutionary relationships between CmoMYB and AtMYB proteins. Full-length CmoMYB and AtMYB protein sequences were implemented for multiple sequence alignment using the ClustalW Program in the software MEGA, and the self-help method of phylogenetic experiments (following parameters: *p*-distance, partial deletion, and bootstrap=1000) was used to construct the neighbor-joining (NJ) phylogenetic tree. The tree was visualized and optimized through the ChiPlot (https://www.chiplot.online/#Phylogenetic-Tree).

For the duplication analysis of the pumpkin *MYB* genome, we employed BLAST to compare the genomic sequences and retrieved essential genomic features such as chromosome length, position, inter-chromosomal linkage, and gene annotations from the GFF3 files. The integration of these files, along with the gene link format file, GFF file format file, and Chr-Layout format file was performed using the File Merge function of the MCScanX package in TBtools (v1.108) to investigate *MYB* gene duplication events in pumpkin.

2.3. Chromosomal location, genetic structure, conserved motifs, and cis-acting regulating element prediction

To investigate the genetic structure and chromosomal localization of the *MYB* gene, gff3 files about *C. moschata* were acquired from the Cucurbitaceae database. Subsequently, the conserved motifs within CmoMYB proteins were delineated utilizing the Multiple Em for Motif Elicitation (MEME) Program (https://meme-suite.org/meme/tools/meme.cgi), with a specified parameter setting of 10 as the maximum number of conserved motifs to be identified. Furthermore, the 2 kbp promoter sequences associated with CmoMYB genes were obtained. The PlantCARE database website (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) served as the platform for the prediction of *cis*-acting elements. TBtools software (v1.108) was used to visualize the above results.

2.4. Plant materials, abiotic stress, and hormones treatment

The expression levels of *CmoMYBs* were investigated under three hormones and abiotic stress, including ABA, SA, JA, and simulated drought. The ‘TianMiyihao’ was used as the experimental material. Seeds were sterilized with 75% ethanol, then rinsed five times with sterile water and germinated on Petri dishes. After three days, seedlings with good growth conditions were cultured with Hoagland's nutrient solution in a greenhouse (28℃, 70–80% humidity, 16 h light/8 h dark). The roots, stems, and leaves of pumpkin were harvested to analyze the expression

levels of *CmoMYB* genes. Meanwhile, uniformly sized three-leaf seedlings were treated with simulated drought treatment (20% PEG6000) and different hormones (100 μM JA, 10 μM ABA, and 100 μM SA). Leaves from each group were obtained after 0, 3, and 6 h of treatment, respectively. The samples were snap-frozen in liquid nitrogen and stored at −80℃. Each sample consisted of three different plant leaves and three experiments were performed.

2.5. RNA extraction, cDNA synthesis, qRT-PCR, and Expression analysis

Total RNA from each sample was extracted by using Trizol reagent (Takara, Beijing, China), and the cDNA was synthesized by using the First-Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). The specific primers for the *ComMYB* genes were designed using Primer Premier 5 software (Table S2) for qRT-PCR analysis. The reaction consisted of 10 μL AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China), 0.2 μmol L^{-1} upstream and downstream primers, 2.0 μL cDNA, and up to 20 μL with ddH_2O. The qRT-PCR process was set as follow steps: 95 ℃ for 3 min, followed by 40 cycles of 95 ℃ for 10 s, 60 ℃ for 30 s, and 60 ℃ for 30 s. The Cmoβ-actin gene was used as the normalization reference gene. The relative expression level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. Three experimental replicates were performed for each sample. For tissue-specific expression analysis of 175 *CmoMYB* genes, the expression data for four tissues (leaves, roots, stem, and fruit) were obtained from the Cucurbitaceae database [31]. The TBtools software was used to construct a heatmap for specific expression analysis.

3. Results

3.1. Identification of the CmoMYB gene family

To identify members of the *CmoMYB* gene family, we conducted a comprehensive search of the entire set of *Cucurbita moschata* protein sequences to identify proteins harboring either MYB or MYB-like domain sequences, which served as initial candidate proteins. Subsequently, candidate proteins underwent further validation through the HMMER website, with proteins containing divergent conserved domains being excluded from the analysis. A total of 175 CmoMYB proteins were successfully identified and individually designated. Detailed information regarding the *CmoMYB* genes is provided in supplementary Table S1. As shown in Table S1, a total of 175 CmoMYB proteins were successfully identified (see Table S1 for details), including 1R-MYB (21), R2R3-MYB (149), 3R-MYB (4), and 4R-MYB (1). The lengths of the CmoMYB sequences ranged from 249 to 3,117 base pairs (bp), corresponding to polypeptide lengths

spanning from 82 (CmoMYB171, CmoMYB172) to 1,038 (CmoMYB150) amino acids, with an average length of 343 amino acids. Furthermore, the calculated molecular weights (Mw) of the CmoMYB proteins ranged from 9.80 to 114.86 kilodaltons (kDa), while the theoretical isoelectric points (pI) ranged from 4.61 (CmoMYB30) to 11 (CmoMYB163). In addition to being distributed in the nucleus, some proteins were also distributed in the cytoplasm (Table S1).

To explore the orthologous relationship between CmoMYB proteins and their counterparts in *Arabidopsis thaliana* (AtMYB proteins), we employed the neighbor-joining method to construct a phylogenetic tree (Figure 1). Analysis of the phylogenetic tree revealed the classification of MYB proteins from both species into 33 distinct categories. Among these, 126 AtMYB proteins were allocated into 25 subgroups. Notably, the majority of CmoMYB proteins (depicted by purple circles in Figure 1) clustered together with their homologous AtMYB proteins (marked by green circles in Figure 1), indicating evolutionary conservation. However, exceptions were observed in clades C16 (comprising CmoMYB64, CmoMYB74, and CmoMYB159) and C24 (encompassing CmoMYB4, CmoMYB5, and CmoMYB6), which exclusively contained CmoMYB proteins. This observation suggests that MYB proteins may have undergone distinct evolutionary adaptations to environmental changes.

3.2. Chromosomal localization

The 175 CmoMYB genes were mapped to the twenty chromosomes of Cucurbita moschata, with varying gene distributions across each chromosome. The distribution patterns of *CmoMYB* genes on chromosomes revealed that chromosome 14 harbored the highest number of *CmoMYB* genes (20), followed by chromosome 1 (17), and the least number of *CmoMYB* genes were found on chromosome 16 (3). Additionally, there were 14 genes each on chromosomes 2, 4, and 11, respectively; 6 genes each on chromosomes 5 and 19, respectively; 5 genes each on chromosomes 3, 7, 8, 9, and 17, respectively; and 2 genes each on chromosomes 10 and 12, respectively. Furthermore, chromosomes 6, 13, 15, 18, and 20 contained 10, 8, 11, 4, and 9 genes, respectively. Several *CmoMYBs* are clustered in different specific locations, such as the top of chromosomes 1 and 14, and the bottom of chromosomes 4 and 11. For the selection of duplicated *MYB* gene pairs, *MYB* duplicates were scrutinized within the pumpkin genome, resulting in the detection of 135 homologous *MYB* gene pairs distributed across 20 chromosomes (refer to Figure S2 and Table S3). Among these, 142 genes exhibited one to four homologous MYB counterparts. Such as *CmoMYB8* and *CmoMYB50* both have four homologous *MYB* genes.

3.3. Analysis of genetic structure and conserved domains of CmoMYBs

The genetic structure and conserved motif composition of *CmoMYB* genes were analyzed for 175 *CmoMYB*s (Figure 2). Analysis of the structural characteristics of these genes revealed that the vast majority (94%, 165) exhibited intron numbers ranging from 1 to 14, with CmoMYB168 presenting the highest count of both exons (15) and introns (14). Conversely, a subset of 11 *CmoMYB* genes possessed a solitary exon, comprising *CmoMYB56*, *CmoMYB126*, *CmoMYB127*, *CmoMYB131*, *CmoMYB132*, *CmoMYB139*, *CmoMYB144*, *CmoMYB148*, *CmoMYB160, CmoMYB165*. The majority (85 of the 175 *CmoMYBs*) of *CmoMYBs* were typically spliced with 3 exons and 2 introns. Moreover, most *CmoMYBs* (85%) had no more than 3 introns.

Employing the MEME tool facilitated the identification of conserved motifs, thus enhancing the comprehension of CmoMYB protein diversity within the pumpkin genome. The findings, illustrated in Table 1, delineated the presence of 10 conserved motifs. Figure 2 depicted that numerous CmoMYBs predominantly comprised motifs 1, 2, 3, 4, 5, 6, and 7/8. While motifs typically occur singularly, exceptions were noted; for instance, motif 3 was repeated in CmoMYB115 and CmoMYB173 but was absent in CmoMYB151. Similarly, motif 4 exhibited duplication in CmoMYB30 but was not detected in CmoMYB159. Moreover, closely related CmoMYB proteins tended to exhibit analogous motifs, suggesting functional similarities within specific subgroups. Notably, certain CmoMYBs displayed unique motifs; for instance, motif 9 exclusively appeared in the C12 and C13 subfamilies. This underscores the potential involvement of specific motifs in executing distinct functions within MYB proteins.

3.4. Cis-acting element prediction of *CmoMYB* gene promoters

To explore the transcriptional regulatory properties of *CmoMYB* genes, cis-acting elements were predicted by using PlantCARE online software (Figure 3). The analysis unveiled a pervasive distribution of stress- and hormone-responsive elements within the promoters of CmoMYB genes, in addition to numerous core cis-elements. Enumeration of distinct cis-elements revealed that abscisic acid-responsive elements (ABRE, 557) were the most prevalent in the *CmoMYBs*' promoters, succeeded by MeJA-responsive elements (TGACG-motif and CGTCA-motif, 547) and salicylic acid-responsive elements (SARE and TCA-element, 124). Furthermore, these promoters encompassed auxin-responsive elements (TGA, AUXRE), drought-induced response elements (MBS), flavonoid biosynthetic regulation (MBSI), low temperature-responsive elements (LTR), defense and stress-response elements (TC-rich repeats), and various other cis-acting elements.

3.5. Expression analysis of the *CmoMYB* genes

Utilizing expression data extracted from the Cucurbitaceae genome database, a heat map delineating the expression patterns of *CmoMYB* genes across four distinct tissues was generated using TBtools (Figure S3). Approximately 90 of these 175 (51.4%) *CmoMYBs* showed the highest expression level in root, 59 (34%) in stem, 37 (21%) in leaf, and 34(19%) in fruit. Besides, a total of 13 *CmoMYB* genes from different subgroups (Figure 1) were selected for expression analysis by qRT-PCR. The expression levels of 13 *CmoMYBs* were performed in three tissues (root, stem, and leaf) at the three-leaf seedlings stage (Figure 4). A variety of expressions of these *CmoMYBs* were found in the three tissues. Most *CmoMYBs* including *CmoMYB99*, *CmoMYB142*, *CmoMYB154*, *CmoMYB144*, *CmoMYB116*, *CmoMYB70*, *CmoMYB46*, *CmoMYB3*, and *CmoMYB9* were significantly up-regulated in the roots, several *CmoMYB* genes including *CmoMYB165*, *CmoMYB142*, *CmoMYB59*, and *CmoMYB29* were highly expressed in the leaves, while *CmoMYB64* and *CmoMYB59* genes were highly expressed in the stem, indicating that these *CmoMYBs* might be involved in the various biological processes in the different tissues.

3.6. The expression levels of *CmoMYB* genes under hormones and abiotic stresses

The expression levels of many *CmoMYB* genes were significantly changed after ABA treatment (Figure 5). ABA treatment for 3 h induced the expression levels of 10 *CmoMYBs* at a degree from 1.23-fold to 65.33-fold, while *CmoMYB99* was not affected, and reduced the expression of *CmoMYB165* and *CmoMYB154* to 77.3% and 63.9%, respectively. The *CmoMYB* genes were significantly regulated after 6 h after ABA treatment (10 up-regulated and 1 down-regulated), except for *CmoMYB64* and *CmoMYB59*, which returned to initial values. As shown in Figure 6, 11 *CmoMYB* genes were significantly regulated at 3 h under JA treatment (9 up-regulated and 2 down-regulated), except for *CmoMYB59* (which started to decrease at 6 h) and *CmoMYB3* (which started to increase at 6 h). The transcription levels of 7 *CmoMYB* genes were less significant at 6 h than at 3 h, such as, the expression levels of *CmoMYB99* decreased from 50.70-fold to 11.93-fold, *CmoMYB116* decreased from 15.94-fold to 7.79-fold, and even *CmoMYB142* and *CmoMYB29* did not change significantly at 6 h. After SA treatment, all *CmoMYB* genes were significantly regulated at 3 h (11 up-regulated and 2 down-regulated) (Figure 7). After 6 h of treatment, the expression level of 12 *CmoMYB* genes started to decrease, except for *CmoMYB144*, which was up-regulated. Compared with 0 h, 8 *CmoMYB* genes had no significant change, only 3 *CmoMYB* genes had a significant change, and 2 *CmoMYB* genes were still continuously down-

regulated at 6 h. Drought treatment for 6 h significantly increased the transcript levels of 10 *CmoMYB* genes (*CmoMYB99*, *165*, *142*, *144*, *116*, *70*, *46*, *59*, *3*, and *9*) with distributions increased by 43.01-, 1.55-, 4.32-, 12.44-, 5.78-, 12.72-, 4.96-, 4.18-, 9.02-, and 9.43-fold, respectively, while *CmoMYB29* decreased to 77%, and *CmoMYB64* and *CmoMYB154* did not change significantly (Figure 8). Interestingly, 7 genes (*CmoMYB165*, *142*, *154*, *70*, *46*, *59*, and *9*) appeared to be down-regulated at 3 h and significantly up-regulated at 6 h.

4. Discussion

The MYB gene family has been systematically characterized across diverse plant species, including Chinese pear, rice, *Arabidopsis*, and soybean [2, 5, 18, 23]. Despite extensive investigations in these species, the *CmoMYB* gene family in *Cucurbita moschata* remains relatively understudied, with its functional roles yet to be elucidated. This current investigation represents the foundational analysis of the MYB superfamily genes within *C. moschata*, leveraging the available genomic resources as detailed by Sun H et al. [31]. A comprehensive survey revealed 175 proteins harboring MYB repeats. The Mw and pI of these proteins emerged as crucial parameters influencing their molecular and biochemical functionalities [48]. Notably, our analysis unveiled substantial variations in both size and pI among CmoMYB proteins, a trend reminiscent of findings reported by Li et al. [32], implying potential context-dependent functional diversity among CmoMYB proteins. Typical TFs encompass four pivotal domains, including nuclear localization signal, DNA-binding, transcriptional regulatory sites, and oligomerization domains [2]. Protein subcellular localization predictions show that all CmoMYB proteins are localized to the nucleus. The chromosomal distribution analysis revealed that *CmoMYBs* are dispersed across the 20 chromosomes of *C. moschata*. however, this distribution appears uneven. Chromosome 14 emerged as the locus harboring the highest number of *CmoMYB* genes, followed by chromosome 1, while chromosome 16 exhibited the fewest *CmoMYB* genes. Furthermore, numerous *CmoMYBs* tend to cluster in distinct genomic regions, displaying notably elevated densities towards the chromosomal telomeres (Figure S1). This pattern of *ComMYB* gene distribution bears resemblance to observations documented in prior studies across other species, such as chili peppers [33] and potatoes [17].

As part of this study, a phylogenetic tree was constructed for CmoMYBs and AtMYBs in order to further investigate their orthologous relationship (Figure 1). The resulting phylogenetic

tree revealed 33 distinct categories. Our findings align with previous research, indicating that members within the same branch share conserved functions, likely stemming from a common ancestor [34, 35]. Most of the CmoMYB proteins were clustered with AtMYB proteins. For example, the CmoMYBs of CmoMYB104, CmoMYB38, CmoMYB36, CmoMYB40, CmoMYB30, CmoMYB102, and CmoMYB26 grouped with AT3G62610, AT2G47460, and AT5G49330 to form clade C26 (The syntenic analysis between these proteins was shown in Figure S4), which may indicated that these CmoMYB genes might related to the biosynthesis of flavonoids [36-38]; clade C11 was consists of CmoMYB83, CmoMYB94, CmoMYB86, and CmoMYB96 of pumpkin and the reported proteins AT3G27810, AT5G40350, AT3G01530 (The syntenic analysis between these proteins was shown in Figure S5), which are involved in the control of PAL genes and the elongation of staminal filaments [39, 40]. Conversely, clades C16 and C24 lacked AtMYBs, indicating that certain CmoMYB genes may be unique to pumpkin. Overall, the clustering patterns offer valuable insights into the roles of CmoMYB proteins. Moreover, the analysis of gene structure and motif protein alignment strongly supports subgroup classification (Figure 2). Consistent with prior studies, our observations indicate that MYB genes within the same subgroup typically exhibit similar exon-intron structures, highlighting their high conservation across species [2, 41].

MYB proteins feature a structurally dynamic region, responsible for regulatory activities, alongside a conserved MYB structural domain, which facilitates the recognition of target gene promoters [42]. Analysis of the motif results showed that CmoMYB proteins belonging to the same subgroup shared the common motifs, suggesting that they might have similar functions. In addition, we analyzed the expression profile of *CmoMYB* genes in different tissues (Figure S3). 90 *CmoMYBs* had the highest level of transcript accumulation in root tissue, 59 *CmoMYBs* in stem tissue, 37 *CmoMYBs* in leaf tissue, and 34 *CmoMYBs* in seed tissue. In the analysis of 13 genes, most *CmoMYBs* in roots were significantly up-regulated, and *CmoMYB29* was expressed only in leaves. In contrast, *CmoMYB64* and *CmoMYB59* genes were highly expressed in stems, suggesting that these *CmoMYBs* may be involved in various biological processes in different tissues, such as root growth, stem elongation, and leaf development (Figure 4).

Cis-acting elements act as sites for specific binding of transcription factors and are involved in the regulation of gene expression [43]. To explore whether the *MYB* gene responds to adversity stress through *cis*-acting elements, we delineated the *cis*-acting elements in the promoter of the

CmoMYBs (Figure 3). Prominently, abscisic acid-responsive elements predominated, succeeded by MeJA-responsive elements, salicylic acid-responsive elements, drought-induced response elements, as well as defense and stress-response elements, among others. The findings imply the potential involvement of the CmoMYB gene family in abiotic stress and hormone-mediated responses. Moreover, the differential transcriptional regulation observed among various *CmoMYB* gene types underscores the diverse functional roles played by CmoMYBs in cellular processes. Guided by these predictions, a subset of 13 *CmoMYB* genes was scrutinized for their responsiveness to three distinct hormones and simulated drought stress conditions. Differential stress responses among these genes were observed, potentially attributed to the distinct repertoire of *cis*-acting elements harbored within their promoters, with notable regulation evident across most *CmoMYB* genes (Figures 3 and 5-8). Furthermore, our inquiry into the responsiveness of these genes to various stressors revealed a spectrum of regulatory patterns, possibly linked to the diversity and abundance of cis-acting elements present in their promoters. Noteworthy prior research highlights the significance of *OsMYB1R1*, *TaMYB31*, *GaMYB85*, and *SsMYB113* in mediating drought stress responses [44-47]. In our investigation, *CmoMYB99*, *CmoMYB144*, *CmoMYB116*, *CmoMYB70*, and *CmoMYB3* exhibited heightened expression under drought stress, contrasting with the down-regulation of *CmoMYB29*. These findings underscore the nuanced transcriptional dynamics under varying stress contexts, indicative of intricate responses mediated by complex signaling pathways [48-51]. Collectively, our results underscore the pivotal role played by the CmoMYB gene family in orchestrating responses to diverse stress stimuli.

5. Conclusions

The MYB superfamily, ubiquitously distributed across eukaryotes, assumes pivotal roles in governing plant growth and development. Notably, environmental stresses, exemplified by drought, constitute significant natural threats to crop plants. The discernment of genes responsive to abiotic stress emerges as a potentially efficacious strategy for augmenting resistance to such adversities in pumpkins. Within the purview of this investigation, a comprehensive analysis of the entire genome identified 175 MYB proteins. While these proteins exhibited a high degree of conservation in motif composition, their expression profiles varied across different tissues, indicative of the nuanced activity and diversity inherent in *CmoMYBs*, governing pumpkin growth. To elucidate the functional attributes of *CmoMYBs*, the prediction of cis-acting elements for these

genes revealed the presence of abiotic and phytohormone-responsive elements within their promoters. This observation suggests a putative role for these genes not only in the regulation of plant growth and development but also in mounting responses to adverse conditions. Delving further into the functional characterization of CmoMYB proteins, we scrutinized the expression patterns of 13 *CmoMYB* genes across distinct branches of the phylogenetic tree under various abiotic stresses, including ABA, SA, MeJA, and drought. The outcomes demonstrated universal responsiveness of all *CmoMYB* genes to these treatments, with discernible variations in expression levels under distinct stress conditions. These findings furnish a foundational framework for prospective inquiries aimed at delineating the functional attributes of the *MYB* gene family within the context of pumpkin physiology.

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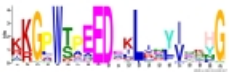
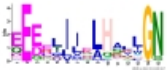
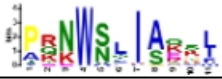
Table 1(on next page)

Table 1. Specific conserved motifs identified by MEME among CmoMYB proteins in *C. moschata*

Motif	Width	Sites	Logo	Consensus sequence	e-value
1	21	171		RCGKSCRLRWINYLR PDJKRG	2.2e-2900
2	21	167		RWSKIAAQLPGRTDN EIKNYW	1.2e-2785
3	21	171		KKGPWTPEEDEKLIN YIQKHG	3.8e-2013
4	15	171		EEEELIIELHALLGN	1.2e-1129
5	11	124		NWRSLPKNAGL	9.8e-566
6	11	85		MGRAPCCDKAG	6.6e-553
7	27	42		NTHJKKKLJKMGIDP VTHKPISDLLDL	1.2e-539
8	8	82		NTHLKKKL	1.2e-160
9	40	9		RTRVQKQAKQLKCD VNSKQFKDTMRYLW IPRLVERIQASS	3.9e-133
10	11	41		PRNWSLIAESJ	7.5e-117

1

Figure 1

Figure 1. The Neighbor-Joining phylogenetic tree of MYB proteins between *C.moschata* and *Arabidopsis*.

The phylogenetic tree was constructed based on the MYB domain alignment by the MEGA7.0 program with a bootstrap test (replicated 1000 times). The proteins are clustered into 33 subgroups (e.g., C1), and 25 clades of AtMYBs are labeled in the evolutionary tree (e.g., S1). Filled purple circles represent the MYB proteins of *C.moschata*, and filled black circles represent the MYB proteins of *Arabidopsis*.

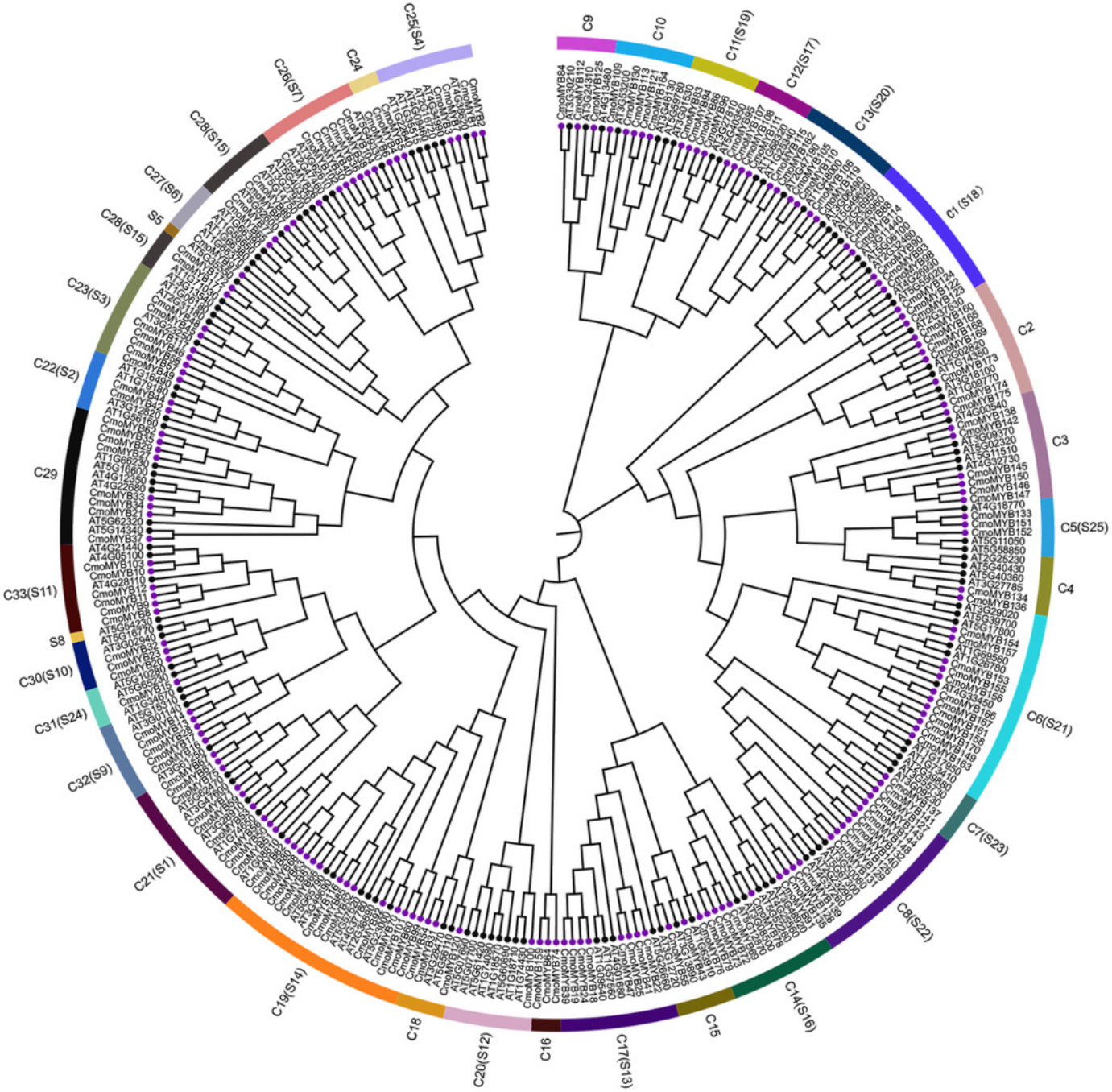

C9
C10
C11(S19)
C12(S17)
C13(S20)
C1(S18)
C2
C3
C5(S25)
C4
C6(S21)
C7(S23)
C8(S22)
C14(S16)
C15
C17(S13)
C16
C20(S12)
C18
C19(S14)
C21(S1)
C32(S9)
C31(S24)
C30(S10)
S8
C33(S11)
C29
C22(S2)
C23(S3)
S5
C28(S15)
C27(S6)
C28(S15)
C26(S7)
C24
C25(S4)

Figure 2

Figure 2. The conserved motifs and exon/intron structures of *MYB* genes.

(A) The distribution pattern of conserved motifs in the CmoMYBs was identified by the MEME web server. Motif distribution includes different colored boxes, each representing a unique numbered motif as indicated in the legend. The width differences among the boxes represent the motif length. (B) Exon/intron structures of *MYB* genes from *C. moschata*. TBtools software was used to visualize the above results. The exons and introns are presented as filled blue sticks and thin gray single lines, respectively. Upstream and downstream regions are represented by black bars at the two ends of sequences.

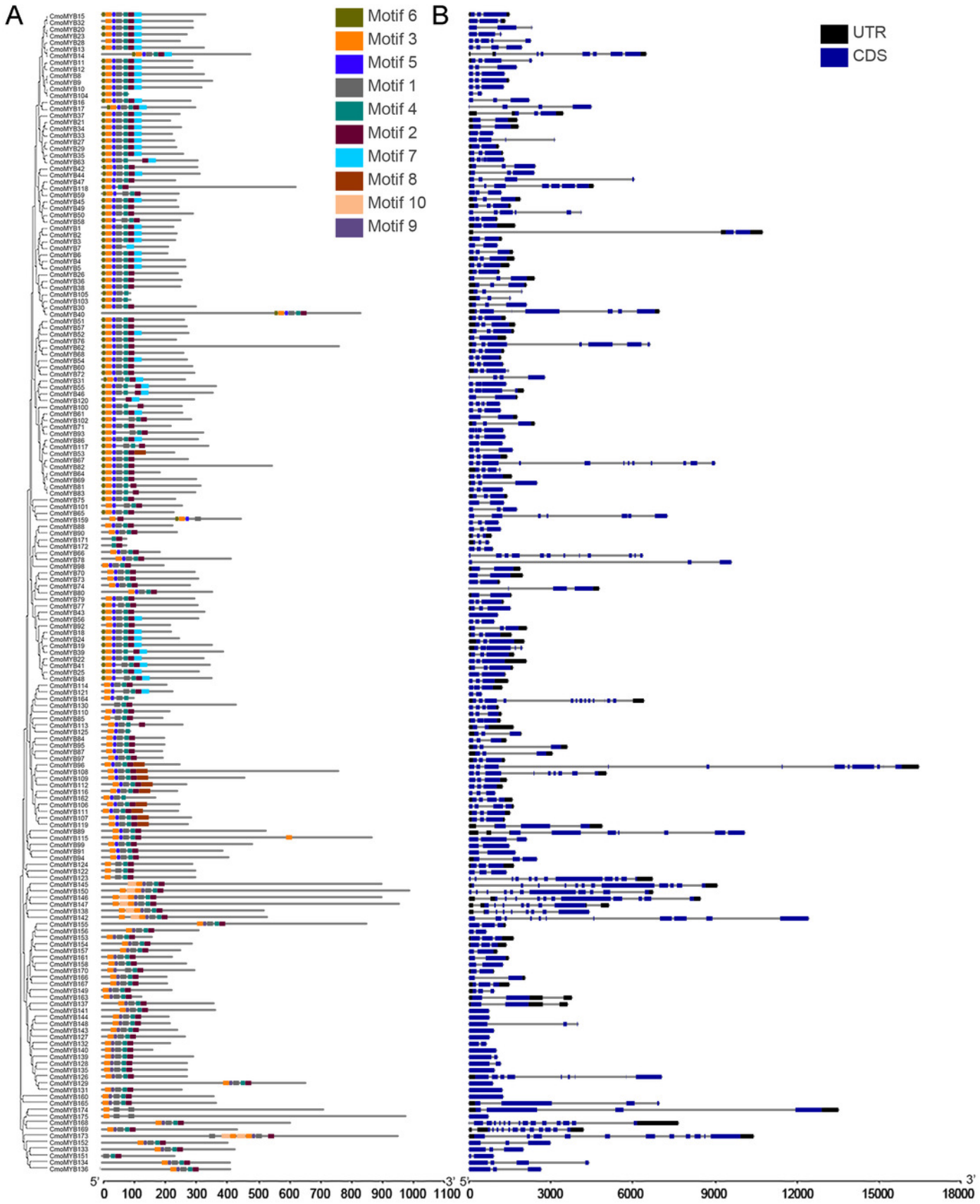
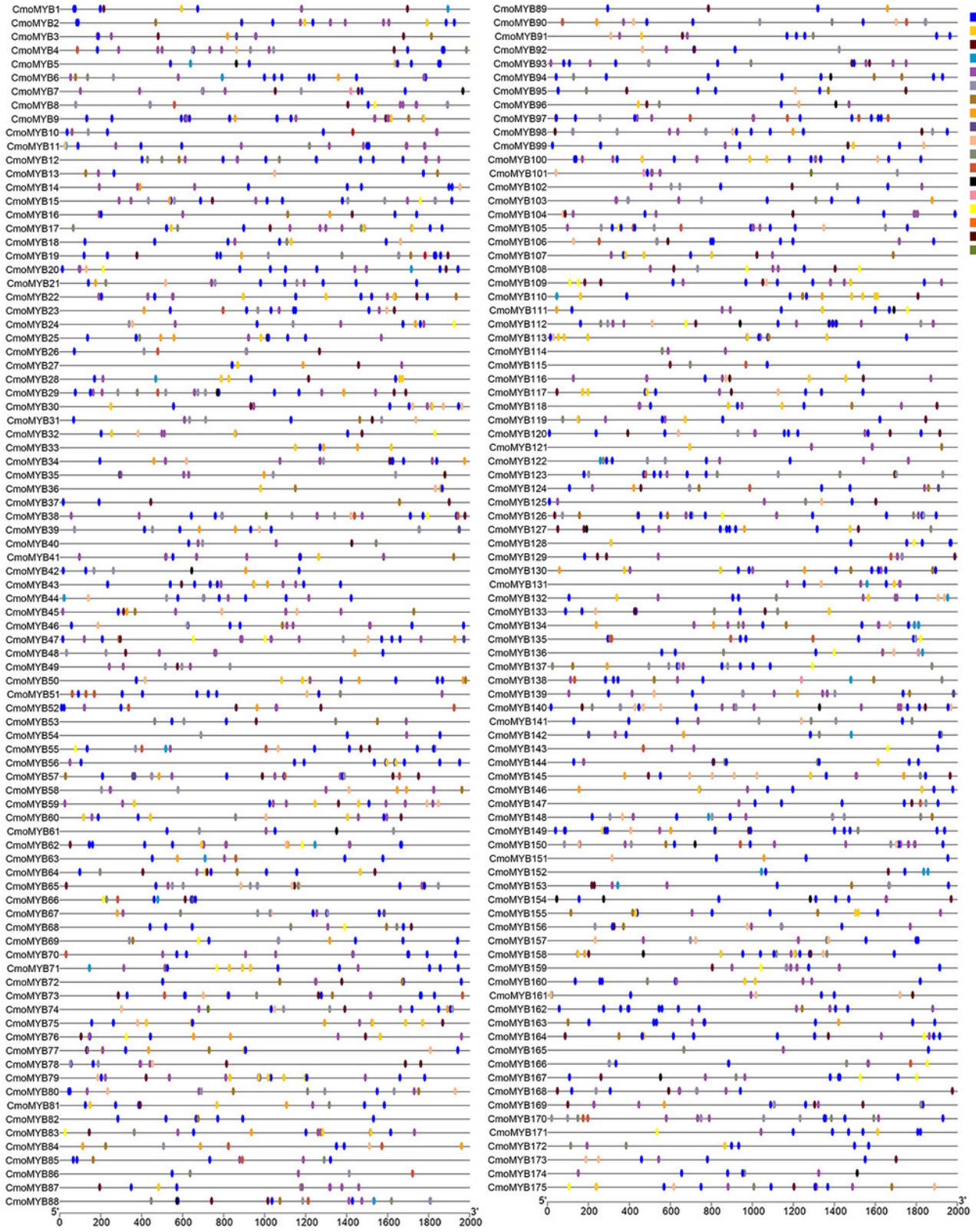

A
Motif 6
Motif 3
Motif 5
Motif 1
Motif 4
Motif 2
Motif 7
Motif 8
Motif 10
Motif 9
5'
3'
0
100
200
300
400
500
600
700
800
900
1000
1100
B
UTR
CDS
5'
3'
0
3000
6000
9000
12000
15000
18000

Figure 3

Figure 3. Cis-acting elements in the promoter region of *ComMYB* genes.

The cis-acting elements were identified by PlantCARE and visualized using the TBtools software. Different colors of the box indicate different cis-acting elements.


CmoMYB1
CmoMYB2
CmoMYB3
CmoMYB4
CmoMYB5
CmoMYB6
CmoMYB7
CmoMYB8
CmoMYB9
CmoMYB10
CmoMYB11
CmoMYB12
CmoMYB13
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800
1000
1200
1400
1600
1800
2000
3'
CmoMYB89
CmoMYB90
CmoMYB91
CmoMYB92
CmoMYB93
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CmoMYB174
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200
400
600
800
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1200
1400
1600
1800
2000
3'
MYB
CGTCA-motif
TCA-element
CCAAT-box
ABRE
TGACG-motif
CAT-box
TC-rich repeats
GARE-motif
TGA-element
LTR
MBS
MBSI
MSA-like
TATC-box
SARE
AuxRR-core
AuxRE

Figure 4

Figure 4. Expression levels of the selected 13 *CmoMYB* genes in different tissues.

Expression levels of the selected 13 *CmoMYB* genes (*CmoMYB99*, *165*, *142*, *154*, *144*, *116*, *70*, *46*, *64*, *59*,*3*, *29*, and *9*) in different tissues (Student's t-test; **p*<0.05, ****p*<0.001).

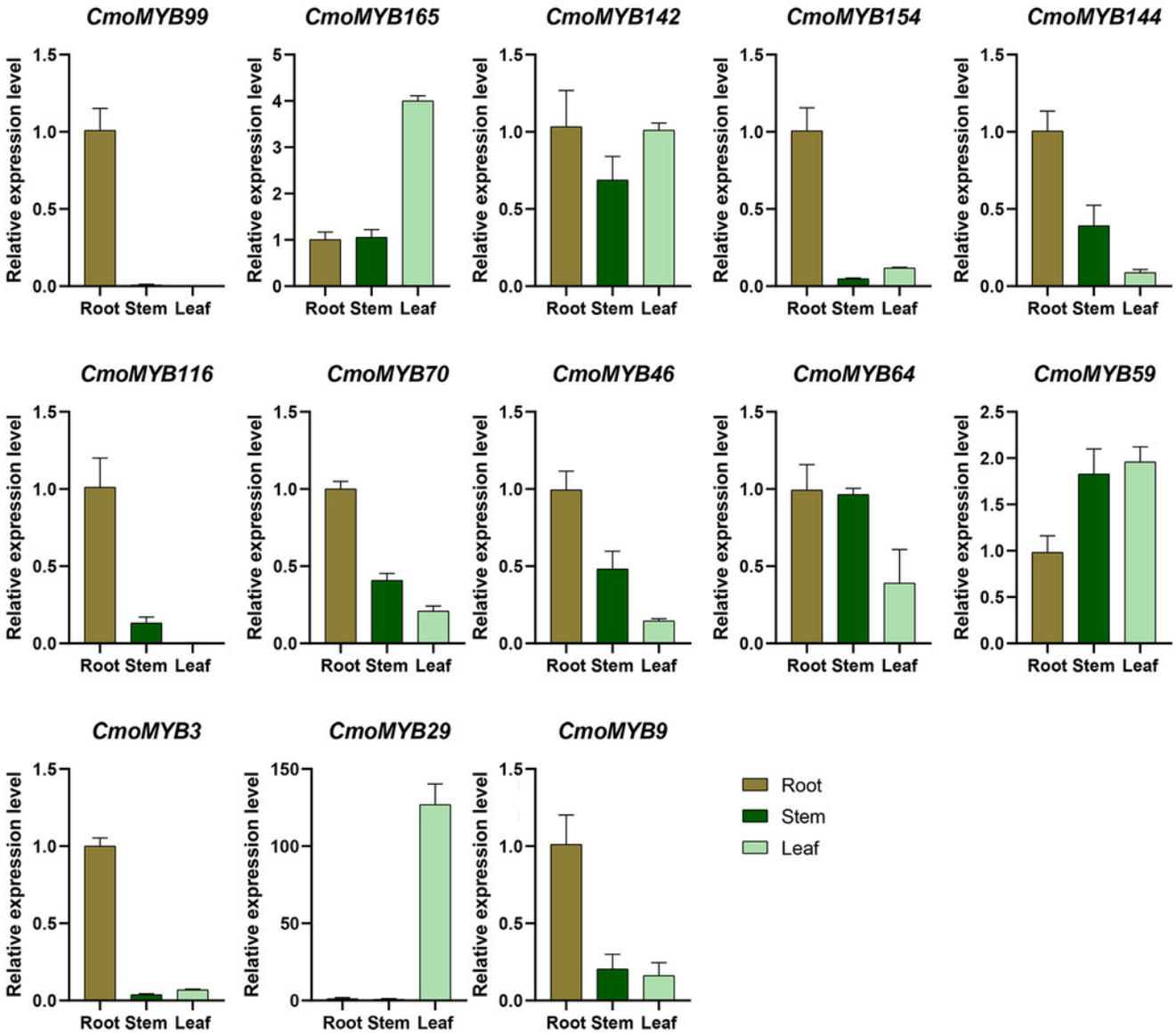

Figure 5

Figure 5. ABA-induced expression patterns of 13 *CmoMYB* genes.

The uniformly sized three-leaf pumpkin seedlings were treated with 10 μM ABA. The leaves were harvested at the indicated times for RNA extraction and qRT-PCR analysis. The *Cmoβ-actin* gene was used as the normalization reference gene. The relative expression level of genes was calculated by the $2^{-\Delta\Delta CT}$ method. Three experimental replicates were performed for each sample (Student's t-test; *p<0.05, **p<0.01, ***p<0.001).

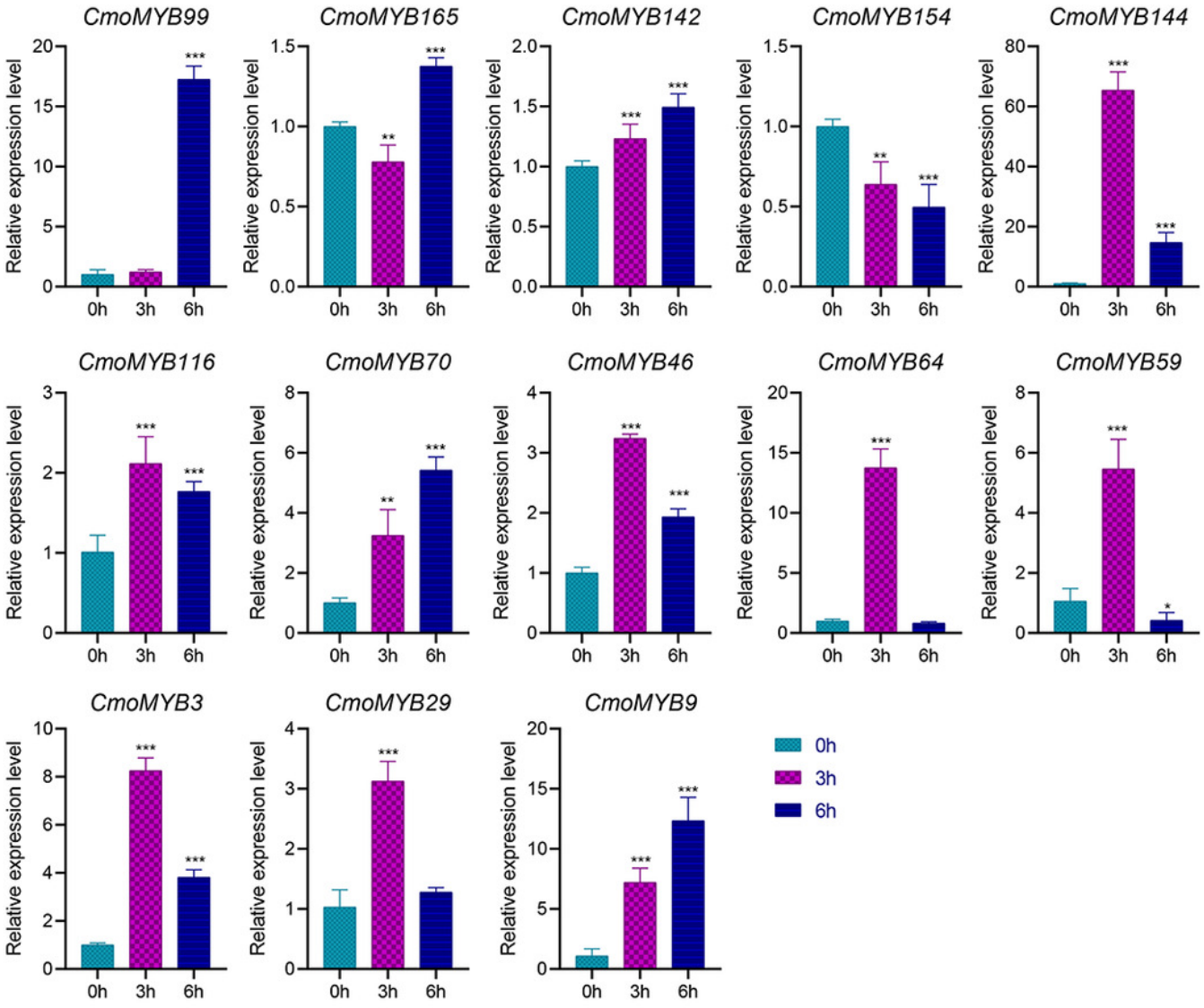

Figure 6

Figure 6. The expression levels of 13 *CmoMYB* genes under JA (100 μM).

The three-leaf pumpkin seedlings leaves were harvested at the indicated times for RNA extraction and qRT-PCR analysis. The *Cmoβ-actin* gene was used as the normalization reference gene. The relative expression level of genes was calculated by the $2^{-\Delta\Delta CT}$ method. Three experimental replicates were performed for each sample (Student's t-test; *p<0.05, **p<0.01, ***p<0.001).

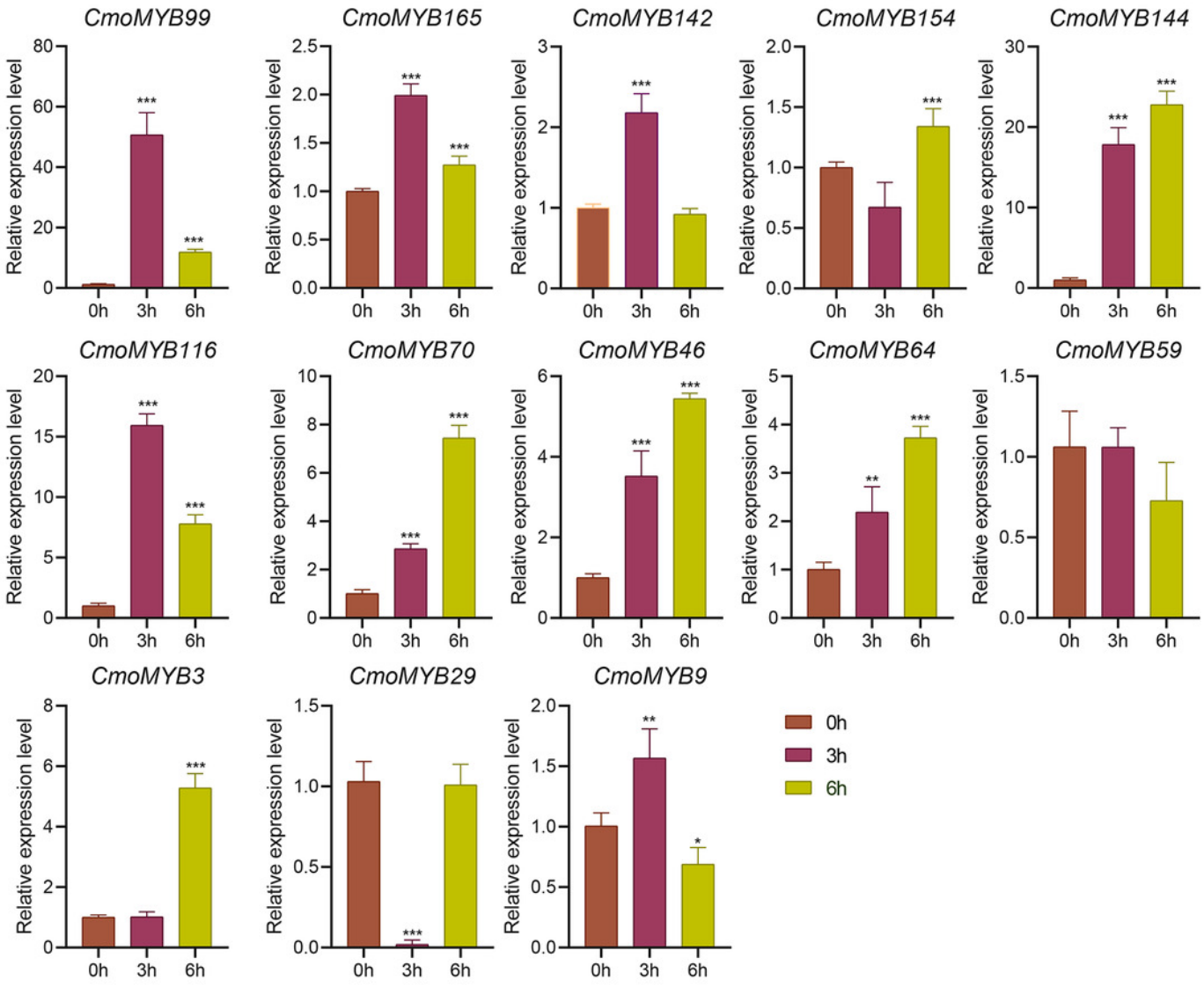

Figure 7

Figure 7. SA-induced expression patterns of 13 *CmoMYB* genes.

The three-leaf pumpkin seedlings' leaves were harvested at the indicated times after being treated with 100 μM SA for RNA extraction and qRT-PCR analysis. The *Cmoβ-actin* gene was used as the normalization reference gene. Three experimental replicates were performed for each sample (Student's t-test; $**p<0.01$, $***p<0.001$).

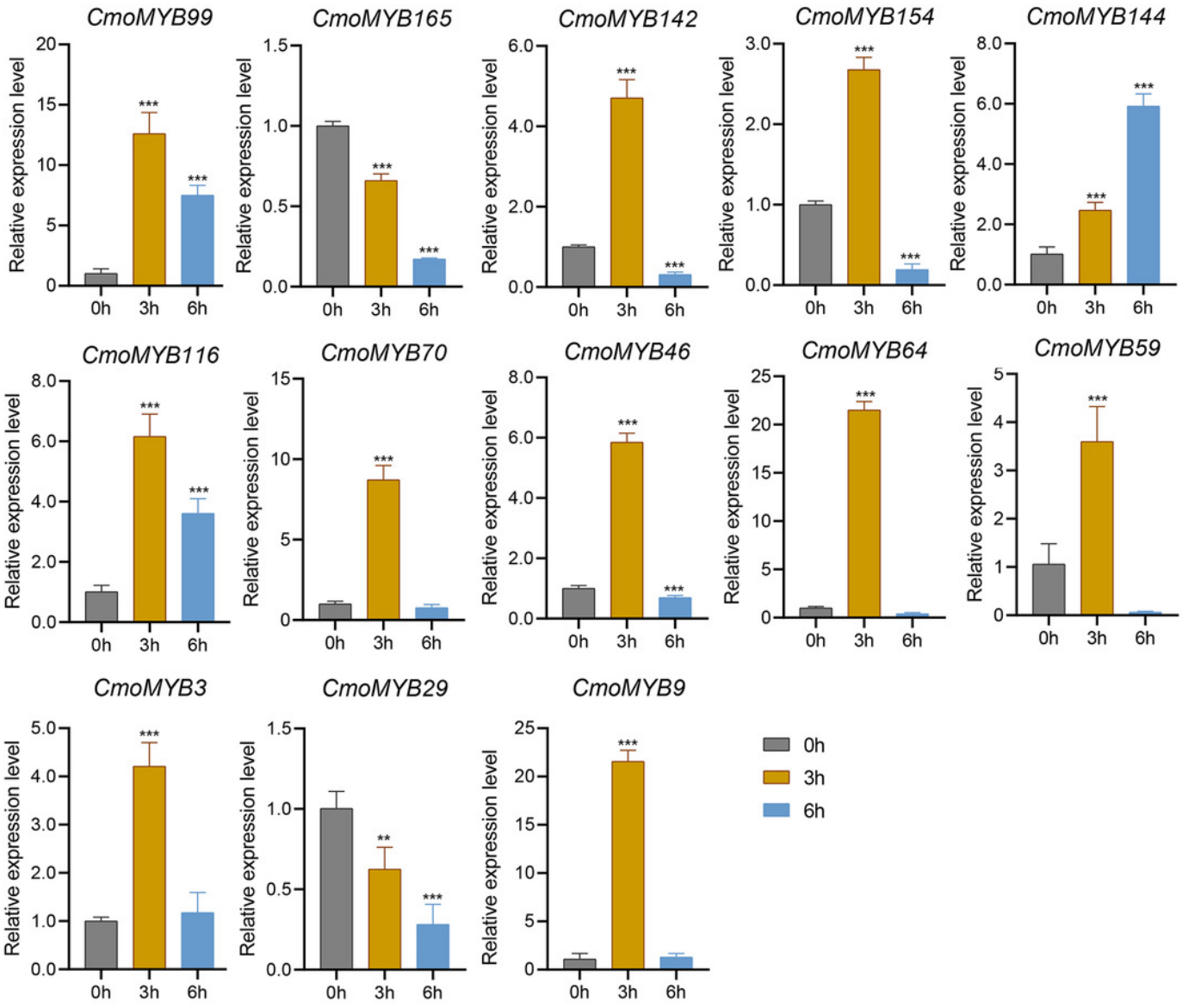

Figure 8

Figure 8. The expression levels of 13 *CmoMYB* genes under simulated drought (20% PEG6000).

The leaves were harvested at the indicated times for RNA extraction and qRT-PCR analysis. The *Cmoβ-actin* gene was used as the normalization reference gene. Three experimental replicates were performed for each sample (Student's t-test; *p<0.05, ***p<0.001).