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IDENTIFICATION AND CHARACTERIZATION OF *MYB* GENES IN *DIMOCARPUS LONGAN* LOUR.

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Abstract

The *MYB* gene family is one of the most widespread plant transcription factor (TF) families, and MYB TFs play key roles in plant development, hormone signal transduction, disease resistance, abiotic stress tolerance and secondary metabolism. Recently, many MYBs have been characterized in various plants. However, little is known about the MYBs involved in secondary metabolite biosynthesis in *Dimocarpus longan* Lour. (*D. longan*). Based on transcriptome data profiling (Accession number: SRP155595), 35 *MYBs* from *D. longan* (*DlMYBs*) were identified. On the basis of their physicochemical properties, phylogenetic relationships, conserved motifs, and tissue-specific expression profiles these *Dimocarpus longan* MYBs (DlMYBs) were analyzed. Fifteen motifs in *DlMYBs* using MEME were found and a phylogenetic tree analysis showed that the DlMYBs identified here were divided into three groups. Group A contained the greatest number (25) of DlMYBs, followed by group B (6) and group C (4). Quantitative real time PCR (qRT-PCR) analysis demonstrated that, of the 35 *MYBs* studied *DlMYB-12* and *DlMYB-22* showed large differences in tissue-specific expression, with both *MYBs* showing very high expression in leaf tissue. These results lay the foundation for further studies of the biosynthesis of secondary metabolites in *D. longan* and further highlight the importance of MYB TFs in plants.

Introduction

The plant *Dimocarpus longan* Lour., commonly called longan, is native to Southeast Asia. It has been widely cultivated in China, Vietnam, Thailand, Australia and Hawaii. Longan production is currently highest in China, accounting for more than 50% of global production (Luo *et al*. 2011). Longan fruit, leaf, flower and seed have been used in traditional Chinese medicines for more than a century (Thitiratsaku and Anprung 2014). Practitioners of traditional Chinese medicine believe that longan can promote blood metabolism, increase immunity, provide insomnia relief, improve learning, and enhance memory. These medical functions are thought to be due to the secondary metabolites - including flavonoids, phenolic acids, and polysaccharides - found in longan (Jiang *et al*. 2009, Chung *et al*. 2010, Prasad *et al*. 2010). In many plants, MYB transcription factor (TF) expression has been found to be related to the biosynthesis of plant secondary metabolites (Dubos *et al*. 2010, Lai *et al*. 2013, Liu *et al*. 2015). This TF family is characterized by the possession of a highly conserved DNA-binding domain in the N-terminal region, which has both DNA binding and protein-protein interaction functions (Feller *et al*. 2011).

Although the functions of MYB TFs have been widely studied in many plant species, to date no studies have examined the molecular mechanisms by which MYB TFs regulate secondary metabolite biosynthesis in *D. longan*. This information may be useful for future studies of pharmacologically useful secondary metabolites of *D. longan*. Therefore, in this study transcriptome

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data for *D. longan* (Accession No.:SRP155595) were used to identify 35 *DlMYBs* and patterns of *MYB* expression in root, stem, and leaf tissues were investigated. In addition, the physicochemical properties, motif composition, phylogenetic relationships of the *DlMYBs* identified here were analyzed. It is expected that the present findings will be useful for further studies of secondary metabolite biosynthesis in *D. longan* and will enrich the understanding of plant MYB TFs.

Materials and Methods

Growth environment for *Dimocarpus longan* Lour was maintained with a temperature of 25ºC and an ambient humidity level of 50%. Root, stem, and leaf tissue samples from 10 plants were harvested from adult *D. longan* plants after plants had been left to grow for two months.

Data regarding the *D. longan* transcriptome (Accession number: SRP155595) were obtained from the NCBI. *DlMYBs* were identified based on the NR database annotation and further confirmed using the NCBI Conserved Domain Search tool. Bioinformatics analyses were achieved by ExPasy, SOPMA, MEME and MEGA 5.0.

The cetyltrime thylammonium bromide (CTAB) method was applied to extract total RNA from roots, stems and leaves (Jaakola *et al.* 2001). The TransStart® Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) was used for all qRT-PCR reactions. qRT-PCR specific primers are presented in Table 1. Three experimental replicates were performed for each sample.

Results and Discussion

According to the previously published *D. longan* transcriptome data (Accession number: SRP155595), in total 35 genes were identified as putative *DlMYB* genes based on NR annotation and searches using the NCBI Conserved Domain Search tool. Detailed characterizations of the *DlMYBs* are listed in Table 2. The *MYBs* identified in *D. longan* were named *DlMYB-1* to *DlMYB-35*. The protein length and molecular weight of the DlMYBs ranged from 94 aa to 1039 aa and from 10632.19 Da to 114615.32 Da, respectively. These imply that different DlMYBs might function in diverse microenvironments. A protein whose instability index is smaller than 40 is predicted to be stable, while values above 40 predict protein instability (Guruprasad *et al*. 1990). The predicted instability indexes of the DlMYB proteins identified here were all greater than 40, suggesting that they have low stability. The maximum and minimum GRAVY scores were −0.521 of DlMYB31 and −1.053 of DlMYB-21, respectively. In *Arabidopsis*, a very few MYB TFs have been identified whose GRAVY scores are positive, suggesting the existence of insoluble MYBs (Katiyar *et al*. 2012).

MEME was used to characterize the motif compositions of the 35 DlMYBs. In total, 10 conserved motifs were identified (Fig. 1); these are displayed below in the positions in which they were found in different DlMYBs (Fig. 2). In general, it was found that DlMYBs showed recent, common evolutionary origins - as determined by the authors phylogenetic trees - possessed similar motif compositions. Motifs 1 and 2 were present in 35 and 33 DlMYBs in the N-terminal region, respectively. Theses two motifs may be related to DNA binding, since this is both an important common feature of MYB transcription factors (Shi and Xie 2014, Roy 2016).

In order to study the evolutionary relationships among the 35 DlMYBs, the author downloaded protein sequence data for 116 *Arabidopsis* MYBs from The Arabidopsis Information Resource (TAIR) and used this data to construct a phylogenetic tree. In general, highly related genes show similar gene structure, length, and amino acid motif composition (Chen *et al.* 2013). The present analysis of 116 random AtMYBs and the 35 DlMYBs identified here resulted in the construction of a comprehensive phylogenetic tree. It was found that the MYBs could be divided into three groups, which are A, B and C (Fig. 3). The group A showed a higher degree of

evolutionary branching than the other two groups, which suggests that its own evolutionary history is complex and deserving of attention in future studies of MYB structure and function. These results depict the predicted phylogenetic tree of MYBs for *D. longan* and *Arabidopsis*.

Table 1. Primer sequences for qRT-PCR.

Gene	Primer sequence	Gene	Primer sequence
DlMYB-1	QF: CAATTTCCGGCCACTTCTGC QR: TCCCCAGCCGATCTAGTGAA	*DlMYB-19*	QF: GGTCACTACTGCAGGCAACA QR: CGAAGCGGTTTTCGAGCATT
DlMYB-2	QF: CCTGCTTTGACTGCAATTGTCT QR: GGGAAGTGAACAAGGGAGCA	*DlMYB-20*	QF: CCCAGACAGTTCTGGTCCAC QR: ACTAGTGCGATGTGCAGCTT
DlMYB-3	QF: TCCCACGAAAGCCTTCAACA QR: TTGAAAACCGACGGCCAAAC	*DlMYB-21*	QF: TGATTTCGTTGTCGGTCCGT QR: GAGGCCTGACATCAAGAGAGG
DlMYB-4	QF: CACAGGTTTTTGGCTTGCCT QR: GAACTTGCAAAGCCTCCACC	*DlMYB-22*	QF: GCTGCTGACTTTGGCTTGAG QR: GAGAGAGACCAACGGAGACG
DlMYB-5	QF: AGGACTTCGGGCTTGGTTTT QR: GTCAACGGCAACCATGTCAC	*DlMYB-23*	QF: TGTTGCATACCTGTTCCCCA QR: GAGTTGCAGACTTCGCTGGA
DlMYB-6	QF: GCTACCTCGAGACTCATGGC QR: ACGGCAACTTTTGCCACATC	*DlMYB-24*	QF: GGTGGTTTGAGTGGTTGACG QR: TGGGCTCAAATAGCCAAGCA
DlMYB-7	QF: AGACAACGTTGGCAACCAGA QR: GTTGGGCATCCGTTGAGAGA	*DlMYB-25*	QF: TCGGTTGGTGAGTCAGTTCG QR: GATTACACCACCGGAGTCGG
DlMYB-8	QF: GGAGTGAAATTTCCGCGCTT QR: TGCTTGCAAAACGTTCAGGG	*DlMYB-26*	QF: AACATGGTCACGGTAGCTGG QR: AGCCTACAACTTTTTCCGCA
DlMYB-9	QF: CCTGTTCCCAAGAACCTCGT QR: AGTTCCCAAGCTTGCTGGTT	*DlMYB-27*	QF: CGGCAACTCTTCCCACATCT QR: TTGGTCACCCGAAGAAGACG
DlMYB-10	QF: TCTGAAGGTGCCATGTGGTG QR: ACACCATTCCTCCTTGGAACC	*DlMYB-28*	QF: ATCCCCATCCATGCCGTTTT QR: CATGCTCAATCACGGGCAAC
DlMYB-11	QF: CCAAGAAGGCCAGCTAGTCA QR: GGCTGCAACTCCCATCAGAA	*DlMYB-29*	QF: CCCAGGTCTGCAGGTTTGAA QR: TGCCTTCCTCCTGAGGTGTT
DlMYB-12	QF: CCTGGTATCAAGCGCGGTAA QR: GAAGCTATGGCTGCCCATCT	*DlMYB-30*	QF: AGCAGGCGGTGGAGTCTTAT QR: GAAGAGTTGTAGGCTGCGCT
DlMYB-13	QF: CACCACCGTAACCGACAAGA QR: CGGATCAAGGGACCATGGAG	*DlMYB-31*	QF: GAGACCATCATACGCGCTCA QR: GCACTTGCGTTTGAGAGTGG
DlMYB-14	QF: GGTCTTCCTGCTTCGACCAA QR: GGTCTTCCTGCTTCGACCAA	*DlMYB-32*	QF: GCAAGCTTGGGAACCATTCG QR: GGGAAGGCAACCTTGTTGTG
DlMYB-15	QF: TGGGAAAAGTTGCAGGTTGAG QR: ACCACCTGTTACCCCACTTG	*DlMYB-33*	QF: TTCGGTGTCTGAAGGCAGTG QR: ACCACCTTTTGCTCGCCTTA
DlMYB-16	QF: CCGTCGTTGTGGCAAAAGTT QR: TGTTGCCAAGACGAGCATGA	*DlMYB-34*	QF: TCTGGAGAAATGTTGCCTCTCT QR: CACGGTGAAGGGAAGTGGAG
DlMYB-17	QF: TGGCTTTGAACTGTCGGTGT QR: TCACAAGCCACTTTCCGAGG	*DlMYB-35*	QF: AGTCCCCTTGGTATCCGTCA QR: TTGGCAGCACTTTTCAGCAC
DlMYB-18	QF: GGTCAAGGGACCATGGAGTC QR: TCAAAGACCAGTTCCTCGCC	*Tubulin*	QF: CTCATGTATGCCAAGCGTGC QR: CTCTGCAGACTCAGCACCAA

The relative expression patterns of the 35 *DlMYBs* in different plant tissues were studied. Sixteen *DlMYBs* were found to have different expression levels in root, stem, and leaf tissue (Fig. 4), while the other 19 *DlMYBs* showed no tissue-specific differences. Relatively large differences in gene expression were found for *DlMYB-12* and *DlMYB-22*; their expression levels in leaf tissue were nearly 100- and 200-folds higher than in root, respectively. The 16 *DlMYBs* found to have distinct expression levels by qRT-PCR may be related to critical tissue-specific functions such as development, metabolism, and response to biological stresses. In *Arabidopsis*, *AtMYB94* was found

Table 2. Physicochemical properties of *DlMYBs*.

Gene	Gene ID	ORF (aa)	PI	Aliphatic index	MW (Da)	Instability index (II)	GRAVY	Alpha helix (Hh)	Extended strand (Ee)	Beta turn (Tt)	Random coil (Cc)
DlMYB-1	MK030143	285	6.33	64.04	32103.89	57.35	-0.747	32.63%	3.16%	5.61%	58.60%
DlMYB-2	MK030144	196	8.66	72.14	22797.76	63.48	-0.993	39.80%	5.61%	6.63%	47.96%
DlMYB-3	MK030145	249	9.14	63.90	28992.44	56.92	-0.929	38.15%	8.43%	4.02%	49.40%
DlMYB-4	MK030146	298	6.27	65.74	33591.65	46.71	-0.699	30.87%	5.03%	4.36%	59.73%
DlMYB-5	MK030147	286	5.32	60.00	32861.25	50.77	-0.982	32.52%	6.99%	4.20%	56.29%
DlMYB-6	MK030148	323	6.79	60.43	35446.50	47.12	-0.621	20.74%	13.31%	9.60%	56.35%
DlMYB-7	MK030149	382	8.78	74.82	43706.80	66.10	-0.801	50.52%	4.97%	4.45%	40.05%
DlMYB-8	MK030150	117	9.74	68.38	13542.22	72.90	-0.974	30.77%	9.40%	11.11%	48.72%
DlMYB-9	MK030151	369	5.86	60.51	41841.49	51.87	-0.666	36.59%	3.25%	4.88%	55.28%
DlMYB-10	MK030152	452	6.92	58.72	50343.56	45.66	-0.801	27.21%	4.65%	4.20%	63.94%
DlMYB-11	MK030153	365	7.05	51.07	40938.30	51.46	-0.809	18.63%	17.26%	5.21%	58.90%
DlMYB-12	MK030154	328	6.40	65.12	36663.67	49.63	-0.780	30.79%	5.18%	1.83%	62.20%
DlMYB-13	MK030155	312	8.32	68.49	34140.01	57.27	-0.654	28.85%	7.37%	2.88%	60.90%
DlMYB-14	MK030156	235	9.06	72.68	26824.34	55.14	-0.955	29.79%	8.94%	6.38%	54.89%
DlMYB-15	MK030157	99	9.18	82.83	11651.40	62.14	-0.821	48.48%	5.05%	11.11%	35.35%
DlMYB-16	MK030158	271	5.05	75.28	30202.85	53.07	-0.633	34.69%	5.17%	5.17%	54.98%
DlMYB-17	MK030159	451	6.73	65.57	49984.53	59.58	-0.665	24.39%	3.55%	3.55%	68.51%
DlMYB-18	MK030160	102	10.01	76.57	11661.24	42.60	-0.767	45.10%	9.80%	9.80%	35.29%
DlMYB-19	MK030161	357	6.14	59.24	40306.84	55.67	-0.702	27.73%	8.96%	5.32%	57.98%
DlMYB-20	MK030162	181	9.94	71.66	21190.39	49.03	-0.862	44.75%	4.97%	8.29%	41.99%
DlMYB-21	MK030163	134	9.82	65.45	15703.94	42.64	-1.053	35.07%	5.97%	9.70%	49.25%
DlMYB-22	MK030164	339	6.42	63.69	37898.04	59.90	-0.830	38.64%	5.31%	3.54%	52.51%
DlMYB-23	MK030165	98	9.54	78.67	11560.51	46.10	-0.638	46.94%	5.10%	12.24%	35.71%
DlMYB-24	MK030166	374	5.57	69.57	42433.18	56.48	-0.577	27.01%	13.64%	5.88%	53.48%
DlMYB-25	MK030167	477	5.42	62.01	51793.31	44.62	-0.661	31.66%	5.45%	3.56%	59.33%
DlMYB-26	MK030168	94	9.46	75.85	10632.19	50.15	-0.650	34.04%	3.19%	10.64%	52.13%
DlMYB-27	MK030169	328	5.93	68.96	37233.35	56.27	-0.697	34.15%	6.10%	3.96%	55.79%
DlMYB-28	MK030170	340	6.42	62.79	37981.56	66.15	-0.682	31.76%	6.47%	5.59%	56.18%
DlMYB-29	MK030171	107	9.66	71.12	12374.09	62.24	-0.797	35.51%	7.48%	12.15%	44.86%
DlMYB-30	MK030172	95	9.83	81.16	10980.78	56.48	-0.595	35.79%	8.42%	16.84%	38.95%
DlMYB-31	MK030173	330	6.11	62.91	34634.38	61.19	-0.521	22.73%	8.48%	4.24%	64.55%
DlMYB-32	MK030174	285	5.25	70.42	33107.82	44.28	-0.949	40.00%	6.67%	5.26%	48.07%
DlMYB-33	MK030175	573	7.86	58.71	63377.55	61.68	-0.851	23.91%	5.93%	3.32%	66.84%
DlMYB-34	MK030176	106	9.71	86.51	12322.23	55.35	-0.840	35.85%	4.72%	13.21%	46.23%
DlMYB-35	MK030177	1039	5.24	63.36	114615.32	56.37	-0.646	24.83%	8.08%	2.02%	65.06%

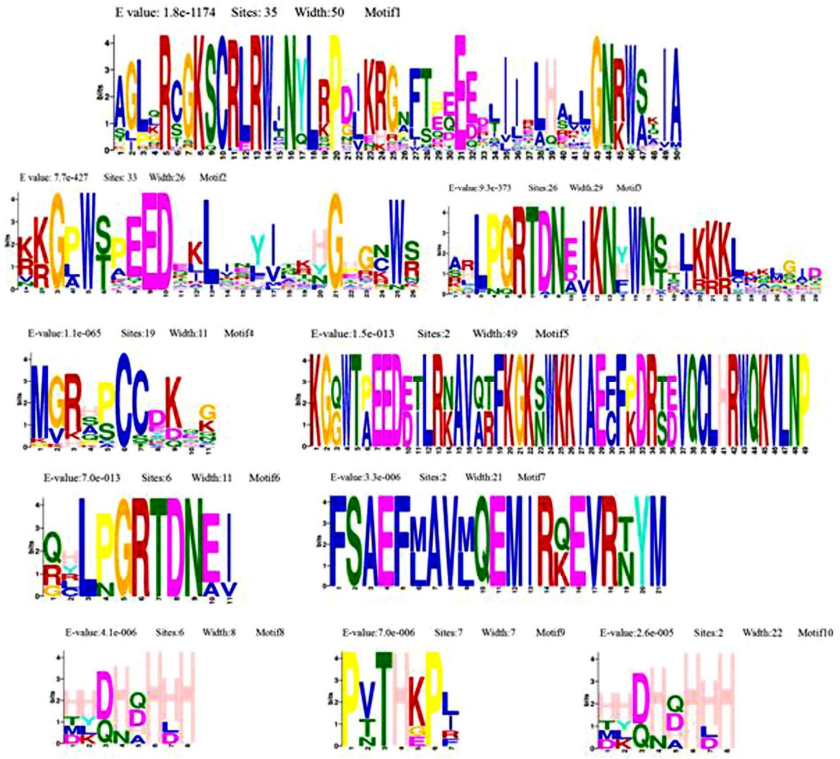

Fig. 1. Motif compositions of *D. longan* MYB TF proteins. E-values represent expected values. Sites indicate how many of the 35 DlMYBs were found to contain the motif. Width indicates the length of the sequence. The height of the letter indicates the frequency of the amino acid.

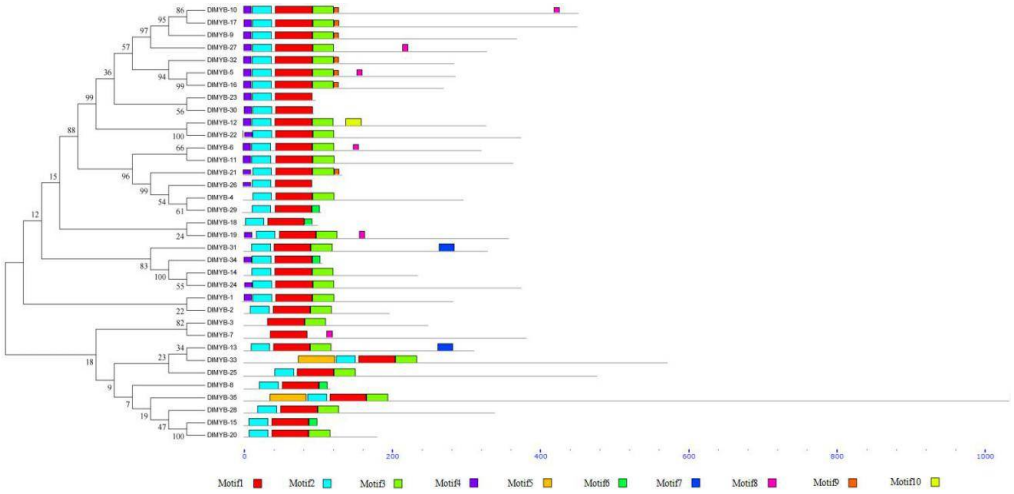

Fig. 2. Phylogenetic relationships and motif distributions across 35 DlMYBs. The phylogenetic tree was constructed using MEGA 5.0. Each colored box represents a motif and non-conserved sequences are displayed in black lines.

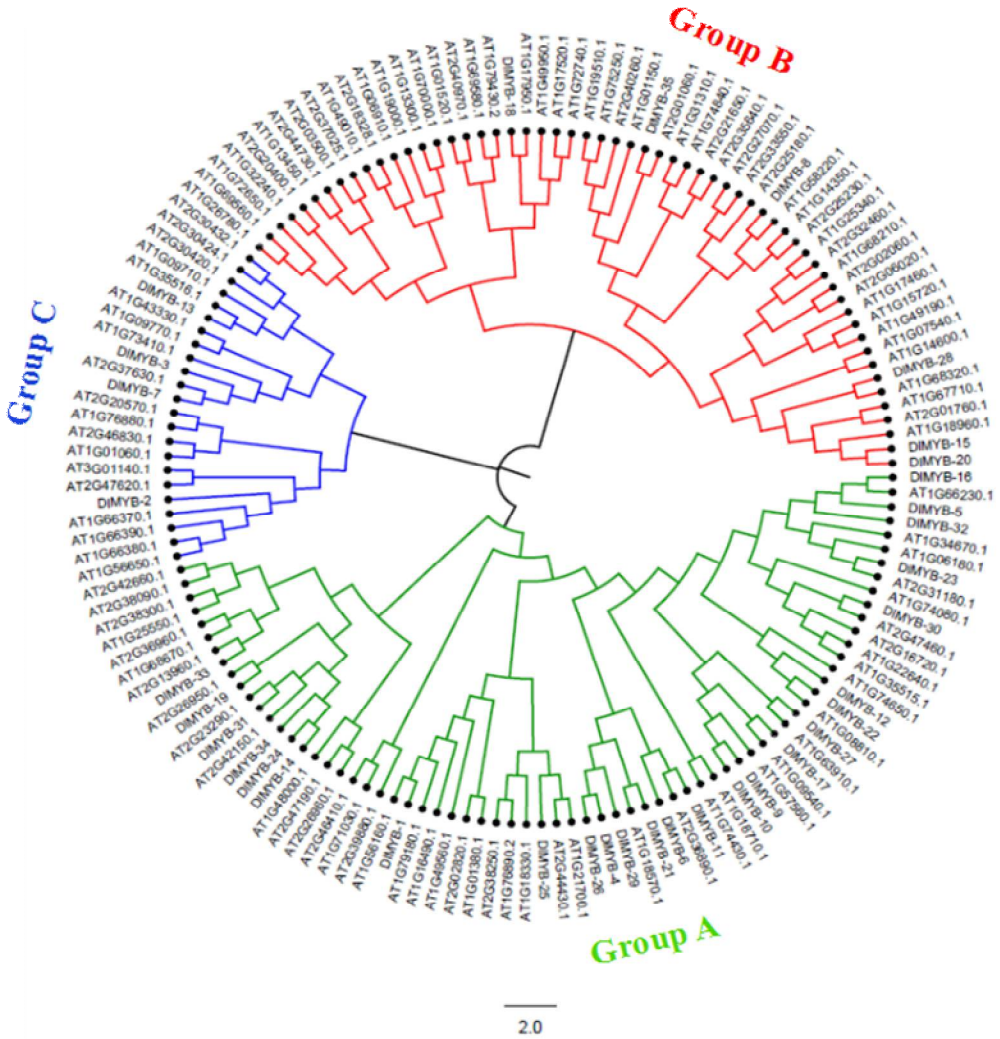

Fig. 3. Phylogenetic tree of DlMYBs from *D. longan* and *Arabidopsis*. 116 protein sequences of *Arabidopsis* MYB proteins were retrieved from TAIR. The phylogenetic tree was constructed using MEGA 5.0 via the neighbor-joining method with 1000 bootstrap replicates. MYBs in groups A, B and C are shown in green, red, and blue, respectively.

to be highly expressed in the epidermis under stress conditions to promote the synthesis of wax in the cuticle, thereby helping plants to cope with drought and salinity (Lee and Suh 2015). Moreover, anthocyanins and flavonols may enhance plant resistance to insect pests, and their biosynthesis in *Arabidopsis* was regulated by *MYBs* (Dubos *et al.* 2010). In addition, the overexpression of *AtMYB75* in *Arabidopsis* has been found to increase the production of anthocyanins and flavonols, presumably to protect against pests (Onkokesung *et al.* 2014). In contrast, overexpression of *AtMYB3*, *AtMYB6* and *AtMYBL2* in *Arabidopsis* led to a measurable reduction of anthocyanin synthesis, suggesting that MYBs can also act as negative regulators of anthocyanin synthesis (Rowan *et al*. 2009). Therefore, it is speculated that the 16 differentially expressed *DlMYBs* may

be related to diverse regulatory pathways involving secondary metabolites in different tissues; further characterization of these pathways should be an important part of future studies seeking to enhance the accumulation of medicinally valuable secondary metabolites produced by *D. longan.*

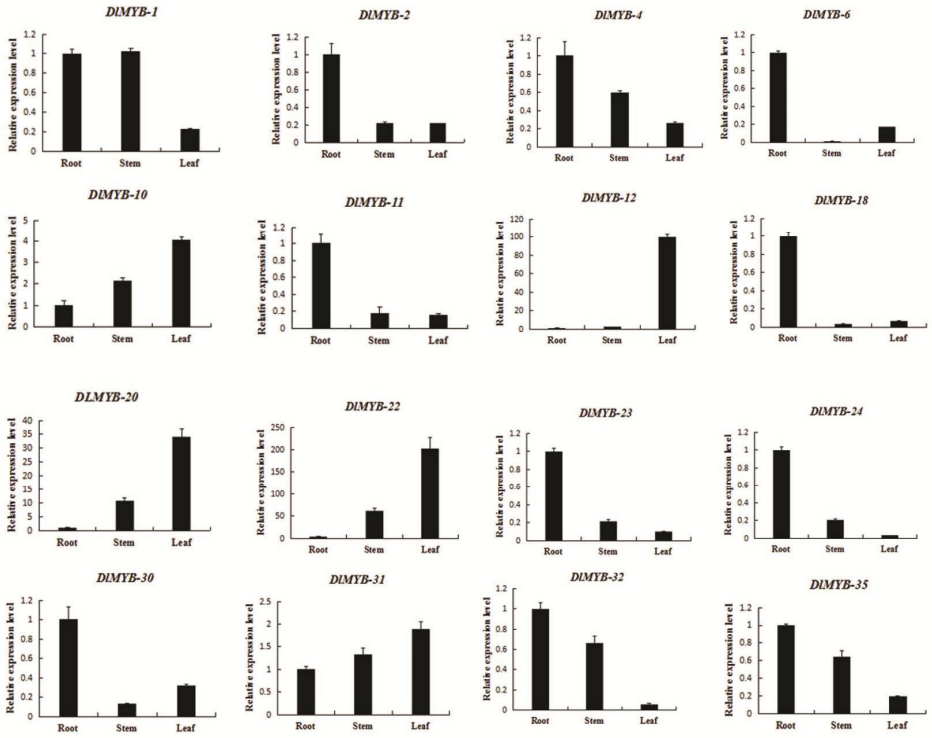

Fig. 4. qRT-PCR results showing the relative expression levels of *DlMYBs* in root, stem and leaf tissue. Error bars represent standard error from three independent replicates.

In this study, 35 *DlMYBs* were identified in *D. longan.* and bioinformatics tools were used to investigate their physico-chemical properties, phylogenetic relationships, and conserved motifs. qRT-PCR showed that 16 *DlMYBs* had different expression levels in root, stem and leaf tissue with *DlMYB-12* and *DlMYB-22* showing the greatest difference in tissue-specific expression. These results may improve the understanding of the biosynthesis of secondary metabolites in *D. longan* as well as the understanding of the diverse functional roles of plant MYB TFs.

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