Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2024**; 29(1): 1
https://doi.org/10.31083/j.fbl2901001

Original Research

Genome-Wide Identification of R2R3-MYB Family Genes and Their Response to Stress in *Dendrobium nobile*

Liping Wu[1,2], Jizhou Fan[1], Xinglong Su[1,3], Daiyin Peng[1,3,4], Shihai Xing[1,3,5,*]

[1]College of Pharmacy, Anhui University of Chinese Medicine, 230012 Hefei, Anhui, China
[2]Department of Pharmacy, Tongling Municipal Hospital, 244000 Tongling, Anhui, China
[3]Institute of Traditional Chinese Medicine Resources Protection and Development, Anhui Academy of Chinese Medicine, 230012 Hefei, Anhui, China
[4]MOE-Anhui Joint Collaborative Innovation Center for Quality Improvement of Anhui Genuine Chinese Medicinal Materials, 230038 Hefei, Anhui, China
[5]Anhui Province Key Laboratory of Research & Development of Chinese Medicine, 230012 Hefei, Anhui, China
*Correspondence: xshshihai@163.com (Shihai Xing)
Academic Editor: Jen-Tsung Chen
Submitted: 26 June 2023 Revised: 12 August 2023 Accepted: 23 August 2023 Published: 9 January 2024

Abstract

Background: *R2R3-MYB* genes comprise one of the largest and most important gene families in plants, and are involved in the regulation of plant growth and development as well as responses to abiotic stresses. However, the functions of *R2R3-MYB* genes in *Dendrobium nobile* remains largely unknown. **Methods**: Here, a comprehensive genome-wide analysis of *D. nobile R2R3-MYB* genes was performed, in which phylogenic relationships, gene structures, motif composition, chromosomal locations, collinearity analysis, and *cis*-acting elements were investigated. Moreover, the expression patterns of selected *DnMYB* genes were analyzed in various tissues and under different abiotic stresses. **Results**: In total, 125 *DnMYB* genes were identified in the *D. nobile* genome, and were subdivided into 26 groups based on phylogenetic analysis. Most genes in the same subgroup showed similar exon/intron structure and motif composition. All the *DnMYB* genes were mapped to 19 chromosomes with the co-linearity relationship. Reverse transcription-quantitative real-time PCR (RT-qPCR) results showed that 8 *DnMYBs* exhibited different expression patterns in different plant tissues, and were differentially expressed in response to abscisic acid, methyl jasmonate, low-temperature stress. **Conclusions**: This work contributes to a comprehensive understanding of the *R2R3-MYB* gene family in *D. nobile*, and provides candidate genes for future research on abiotic stress in this plant.

Keywords: *Dendrobium nobile*; R2R3-MYB transcription factor family; evolutionary analysis; biotic and abiotic stress

1. Introductions

Dendrobium nobile Lindl (family: Orchidaceae), which is a plant with high medicinal value in China and has a medicinal history of more than 1500 years [1]. It is mainly distributed in the subtropical regions south of the Yangtze River such as Guizhou, Yunnan and Guangxi. *D. nobile* contains several classes of important bioactive secondary metabolites including polysaccharides, alkaloids, flavonoids, coumarins, phenols, and sesquiterpenes [2]. Given its medicinal, nutritional, and ornamental functions, *D. nobile* has considerable potential for further development.

Transcription factors (TFs) are localized to in the nucleus and can recognize and bind to *cis*-acting elements in the promoter regions of eukaryotic genes, thereby regulating their expression. Some important TF families in plants include the SBP-box [3], MYB [4,5], and BLH [6,7] families. MYB TFs, characterized by the presence of a highly conserved DNA-binding domain (R) in their N-terminus, comprise one of the largest TFs families in eukaryotes [8]. Depending on the number and position of R sequences, the MYB TF family can be divided into four categories, namely R1-MYB, R2R3-MYB, R1R2R3-MYB, and 4R-MYB [9, 10]. R2R3-MYB is the largest of these subfamilies, and its members have wide-ranging roles in a variety of physiological and biochemical processes, including defenses against biotic and abiotic stresses, metabolism, growth and development [11,12]. The *MYB1* of *Chenopodium glaucum* gene can be induced by salt and cold stress, and its overexpression in *Arabidopsis* significantly enhances salt and cold tolerance in the plant [13]. Meanwhile, the overexpression palm *MYB111* and *MYB157* genes in transgenic *Arabidopsis* plants greatly improves their abiotic stress tolerance [14]. MYB TFs has been identified in a wide variety of plants, including the orchid Dendrobium [8]. For instance, 101 *DoMYB* and 99 *PaMYB R2R3-MYB* genes were identified in the genomes of *Dendrobium officinale* and *Phalaenopsis aphrodite*, respectively [15]. Similarly, 99 R2R3-MYB proteins have been detected in *Dendrobium catenatum* [16]. However, to date, no study has reported on the *R2R3-MYB* gene family in *D. nobile*.

Here, we conducted a genome-wide analysis of the *R2R3-MYB* gene family in *D. nobile*. A total of 125 DnMYB proteins subfamily genes were identified and subjected to phylogenetic, chromosome localization, gene du-

plication, conserved domain and gene structure analysis. In addition, the expression patterns of selected *DnMYB* genes in various tissues and under different stresses were assessed using Reverse transcription-quantitative real-time PCR (RT-qPCR). Our study provides a foundation for future research into the molecular function of *D. nobile R2R3-MYB* genes.

2. Materials and Methods

2.1 Plant Growth and Treatment

The *D. nobile* plants used in this study were cultivated at the Anhui University of Chinese Medicine (Hefei, Anhui, China). *D. nobile* samples are stored in the Herbarium of Anhui University of Chinese Medicine (code: 20220601). The plant was identified as *D. nobile* by Professor Yang Qingshan of Anhui University of Traditional Chinese Medicine. In this study, the root, stem, leaf, and flower of *D. nobile* were tested for gene expression tissue specificity. Each tissue was collected from three different plants, and the collected samples were immediately stored at –80 °C. For the stress treatment, the plants were exposed to 100 µM abscisic acid (ABA), 100 µM methyl jasmonate (MeJA), and low-temperature (4 °C). After treatment, the leaves were collected at different time points (0, 2, 6, 12, and 24 h) and frozen in liquid nitrogen.

2.2 Identification and Sequence Analysis

The genomes of *D. nobile* were downloaded from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genome/?term=dendrobium+nobile) to identify the candidate *R2R3-MYB* genes. The hidden Markov model (HMM) profile of the MYB domain (PF00249) from the Pfam database was used to search the protein sequences [17,18]. The putative DnMYB sequences were checked by the National Center for Biotechnology Information (NCBI). Redundant sequences were removed using CD-HIT online software (http://www.bioinformatics.org/cd-hit/). The remaining sequences were submitted to HMMscan online analysis (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) to determine the presence and quantity of MYB domain, and screen for R2R3-MYB sequences were screened for structural features. A total of 126 R2R3-MYB TF sequences were retrieved from the *Arabidopsis* Information Resource (TAIR) database (https://www.arabidopsis.org/).

The physicochemical properties of the protein sequences were analyzed using the ProtParam tool of ExPASy (https://web.expasy.org/protparam/). TMHMM software (v 2.0) (DTU Health Tech, Lyngby, Denmark) was used to determine transmembrane domain numbers. The subcellular localization of the MYB proteins was predicted using the PSORT tool.

2.3 Multiple Alignment and Phylogenetic Analysis

MEGA X software (https://www.megasoftware.net/) was used for sequence alignment, and a phylogenetic tree based on *D. nobile* and *Arabidopsis* R2R3-MYB proteins was constructed using the neighbor-joining method. The 126 R2R3-MYB protein sequences of *Arabidopsis* were used as references. Bootstrap analysis with 1000 repetitions was performed to compute the reliability of the phylogenetic tree.

2.4 Gene Structure and Conserved Motif Analysis

For gene structure analysis, the *D. nobile* genome sequences corresponding to the *DnMYB* genes were downloaded from NCBI. The Gene Structure Display Server (v.2.04) (http://gsds.gao-lab.org/) was employed to analyze the exons-introns structures of the obtained full sequences. The MEME suite (Multiple Expectation Maximization for Motif Elicitation, version 5.4.1) (https://meme-suite.org/meme/tools/meme) was used for conserved motif analysis [19,20].

2.5 Chromosomal Locations and Synteny Analysis

The physical positions of the *DnMYB* genes on the chromosome were mapped using MG2G online software (http://mg2c.iask.in/mg2c_v2.1/) [21]. The synteny of *DnMYB* genes between *D. nobile* and its homologs in *Dendrobium chrysotoxum* and *Arabidopsis thaliana* was analyzed and visualized using TBtools software (https://github.com/CJ-Chen/TBtools/releases) [22].

2.6 Analysis of the Cis-Regulatory Elements in the Promoters of DnMYB Genes

The sequences of the upstream 2 kb regulatory regions (from the translation start site) of the *DnMYB* genes were obtained from the *D. nobile* genome database, and were analyzed using PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.7 Reverse Transcription-Quantitative real-time PCR (RT-qPCR) Analysis

RT-qPCR analysis was performed using a SuperReal PreMix Plus SyBr Green PCR kit (Qiagen, Shanghai, China) on a Cobas z480 Real-Time PCR System as previously described by Wang *et al*. [23]. Primers were designed with Primer 5.0 (**Supplementary Table 1**). The reactions mixtures contained 2 µL of diluted cDNA, 0.6 µL of each primer, 10 µL of 2 × SuperReal PreMix Plus, and 6.8 µL of RNase-free double-distilled water. The conditions for all the RT-qPCRs were performed as follows: denaturation at 95 °C for 15 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s. Each sample contained three biological replicates. The *18S* housekeeping gene was used as a reference and the relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method [24].

2.8 Statistical Analysis

All experiments were performed with three replicates, and each replicate was repeated three times. The significance of differences in the expression levels of selected 8 *DnMYB* genes was assessed using the student's *t*-test. *p*-value < 0.05 was considered significant.

3. Results

3.1 Identification and Classification of R2R3-MYB Genes in D. nobile

To identify *D. nobile* MYB proteins, the genomic sequence of the plant was downloaded from NCBI. After a searching performed through the Biolinux system using PF00249 as a seed sequence to perform HMMER alignment, the redundant sequences were removed by the CD-HIT online analysis tool, yielding a total of 276 candidate *MYB* genes. The number of MYB domains in the sequence of each candidates was assessed using HMMscan online analysis software, and 125 sequences were screened out that matched the characteristics of *R2R3-MYB* genes. The *R2R3-MYB* genes of *D. nobile* were named *DnMYB1* to *DnMYB125* (**Supplementary Table 2**). These DnMYBs were classified into four distinct subfamilies: R1-MYB (123 proteins, 44.57%), R2R3-MYB (125 proteins, 45.29%), 3R-MYB (27 protein, 9.78%), and 4R-MYB (1 protein, 0.36%). The observation that the R2R3-MYB family encompassed the greatest number of *DnMYB* genes is consistent with that previously reported, namely, that the R2R3-MYB subfamily is the largest subfamily of TFs in plants [25]. The physicochemical properties of the125 *DnMYB* genes, including their chromosomal locus and the length, molecular weight (MW), isoelectric point (pI), number of transmembrane regions, and subcellular localization of MYB proteins, are listed in **Supplementary Table 2**. The lengths of the DnMYB proteins ranged from 88 (DnMYB50) to 2424 amino acids (DnMYB64), with an average number of 348 amino acids. The MW and pI of predicted DnMYB proteins ranged from 10.32 to 270.01 kDa and 4.88 to 10.04. None of DnMYBs were predicted to be transmembrane proteins. Subcellular localization results show that all predicted DnMYB proteins are localized in the nucleus, which is consistent with their functions as TFs [26].

3.2 Phylogenetic Analysis of D. nobile R2R3-MYB Proteins

In order to explore the evolutionary relationship among the DnMYB proteins, a phylogenetic tree was constructed based on 125 and 126 MYB proteins from *D. nobile* and *A. thalian*, respectively (Fig. 1). The results showed most of the DnMYB could be classified into different subclasses, which was supported by high bootstrap values. The phylogenetic tree of the 125 DnMYB were divided into 26 subgroups with the number of members ranging from 1 to 26. The DnMYB proteins has the most members in the S21 subfamily, including 14 DnMYBs. This result is consistent with the classification results of *D. officinale* and *P. aphrodite*. The S14 and S18 subfamily contained 7 DnMYBs respectively, followed by 5 DnMYBs were both divided into S4 and S17 subfamilies. The S13, S22, and S25 subfamilies all contained 4 DnMYBs, while the S1 and S11 subfamilies encompassed 3 DnMYBs each. Four subfamilies, including S2, S6, S10, and S20 all harbored 2 DnMYBs. Each of the remaining subfamilies (S5, S9, S19, S23 and S24) contained only one DnMYBs. Interestingly, no DnMYB proteins were classified into the S12 subfamily. *Arabidopsis* R2R3-MYB TFs are classified into 25 subclasses. Members of subclasses S1, S2, S20, and S22 are involved in regulating plant responses to abiotic stresses [27], while R2R3-MYB TFs within the S4, S5, and S6 subfamilies are involved in plant anthocyanin synthesis [27]. The fact that some AtMYB proteins with similar functions are clustered in the same clade may indicate that DnMYB proteins within a clade may also have similar functions [28]. However, the nature of these functions requires further investigation.

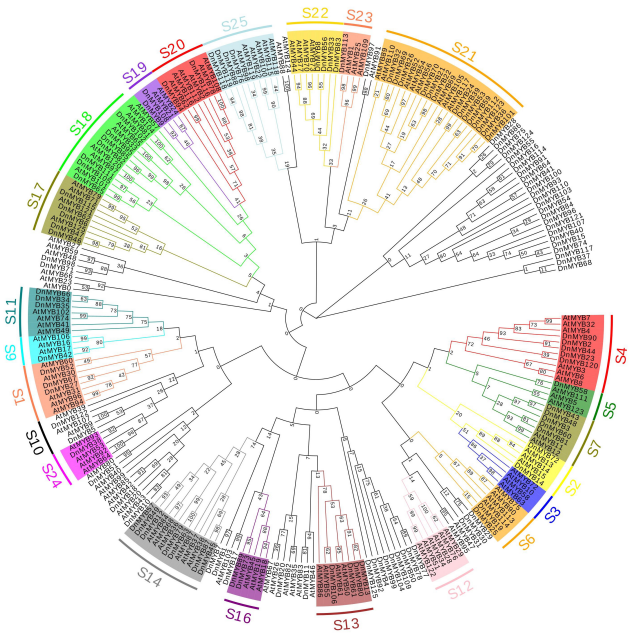

Fig. 1. Phylogenetic tree of R2R3-MYBs in *D. nobile* and *A. thaliana*. The figure was generated by using MEGA X software coupled with a neighbor-joining method and a bootstrap of 1000 replicate.

3.3 Gene Structure Analysis of 125 DnMYB Genes

Exon/intron analysis showed that 125 *DnMYB* genes had introns in their coding regions. The 125 *DnMYB* genes displayed a high degree of diversity in the number and relative positions of introns and exons. It is well-established that genetic structural diversity can explain the evolution of

polygenic families [28]. The gene structure map of these 125 *DnMYB* genes was constructed, and the results showed that the clustering pattern of 125 *DnMYB* genes was not clearly consistent with the exon/intron structure (Fig. 2) (**Supplementary Table 3**). Interestingly, most genes in the same subclass showed similar exon/intron structure, which is in line with that reported for *Liriodendron* [28]. The number of exons ranged from 1 to 13, and most of these genes contained either two (27 genes) or three exons (67 genes) based to the 125 *DnMYB* gene structure.

3.4 Motif Composition of 125 DnMYB Proteins

There are many conserved sequences in MYB TFs and these conserved sequences may combine with some parts of DNA and play a role in expression regulation [29]. To comprehensively investigate the potentially conserved motifs in 125 DnMYB proteins of *D. nobile*, we systematically evaluated their evolutionary relationship and motif composition using MEME online analysis software. A total of 10 motifs were identified in the 125 proteins. Motif 3, located in N-terminal domain, was present in almost all the DnMYBs. Compared with the N-terminus, the C-terminus had greater motif and sequence variability. These differences suggest that different subgroups may have different functions [30] (Fig. 2). Moreover, most of the 125 DnMYBs contained motifs 1, 2, 3, 4, and 6, and most members in the same subgroup harbored more than one identical motif. Motifs 3, 6, and 2 constitute the complete R2 domain, which has three highly conserved tryptophan (W) residues. Motif 1 comprises the complete R3 domain, in which the first tryptophan (W) residue is often replaced by phenylalanine (F), while the remaining two tryptophan (W) residues, which are separated by 19 amino acids, are highly conserved. These characteristics are consistent with those of the MYB domains identified in soybean [30], Ginkgo biloba [31], and *Arabidopsis* [32], among other species. Interestingly, the results also showed that most members of the same subclass have similar motif composition.

3.5 Chromosomal Location and Gene Duplication of the R2R3-MYB Genes

To understand the genomic distribution of the *R2R3-MYB* genes, we employed MG2G online software to identify the chromosomal location of each of the 125 *DnMYB* genes. *D. nobile* has 19 chromosomes (Chr1 to Chr19; **Supplementary Table 4**) and *DnMYBs* were present on all of them, but displayed uneven the arrangement and density (Fig. 3). Chromosome 9 had the highest percentage of *DnMYBs* (12%) and chromosome 4 the lowest. There was a high density of *DnMYBs* located on chromosomes 6, 11, 12, 13, and 17.

A collinearity analysis was undertaken to detect *DnMYBs* gene duplication events. A total of 46 pairs of gene duplications were identified among the *DnMYBs* (Fig. 4). The *DnMYB* genes were homologous to genes in other

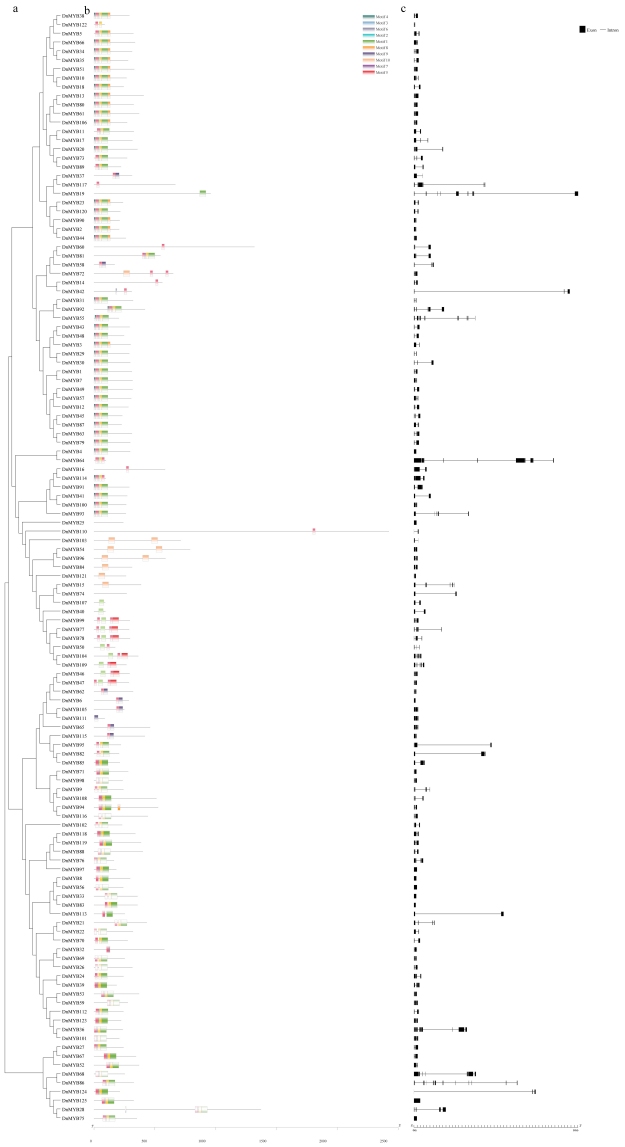

Fig. 2. Phylogenetic relationships, conserved motifs, and gene structures of R2R3-MYBs family member. (a) The phylogenetic tree was constructed based on the full-length sequences of 125 DnMYB proteins using the neighbor-joining method in MEGA X, with 1000 bootstrap replicates. (b) Conserved motifs of DnMYB proteins. Different motifs are represented by various colored boxes. (c) Exon/intron structures of *DnMYB* genes. Exon(s), and intron(s) are represented by black boxes and black lines, respectively.

plants, and syntenic conservation was found among *D. chrysotoxum* (174 orthologous gene pairs dispersed on all chromosomes). 28 collinear gene pairs between *D. nobile* and *A. thaliana* (Fig. 5). The number of orthologous events of *D. nobile-D. chrysotoxum* was far greater than that of *D. nobile-Arabidopsis*, and the closer evolutionary distance between *D. nobile* and *D. chrysotoxum* was confirmed.

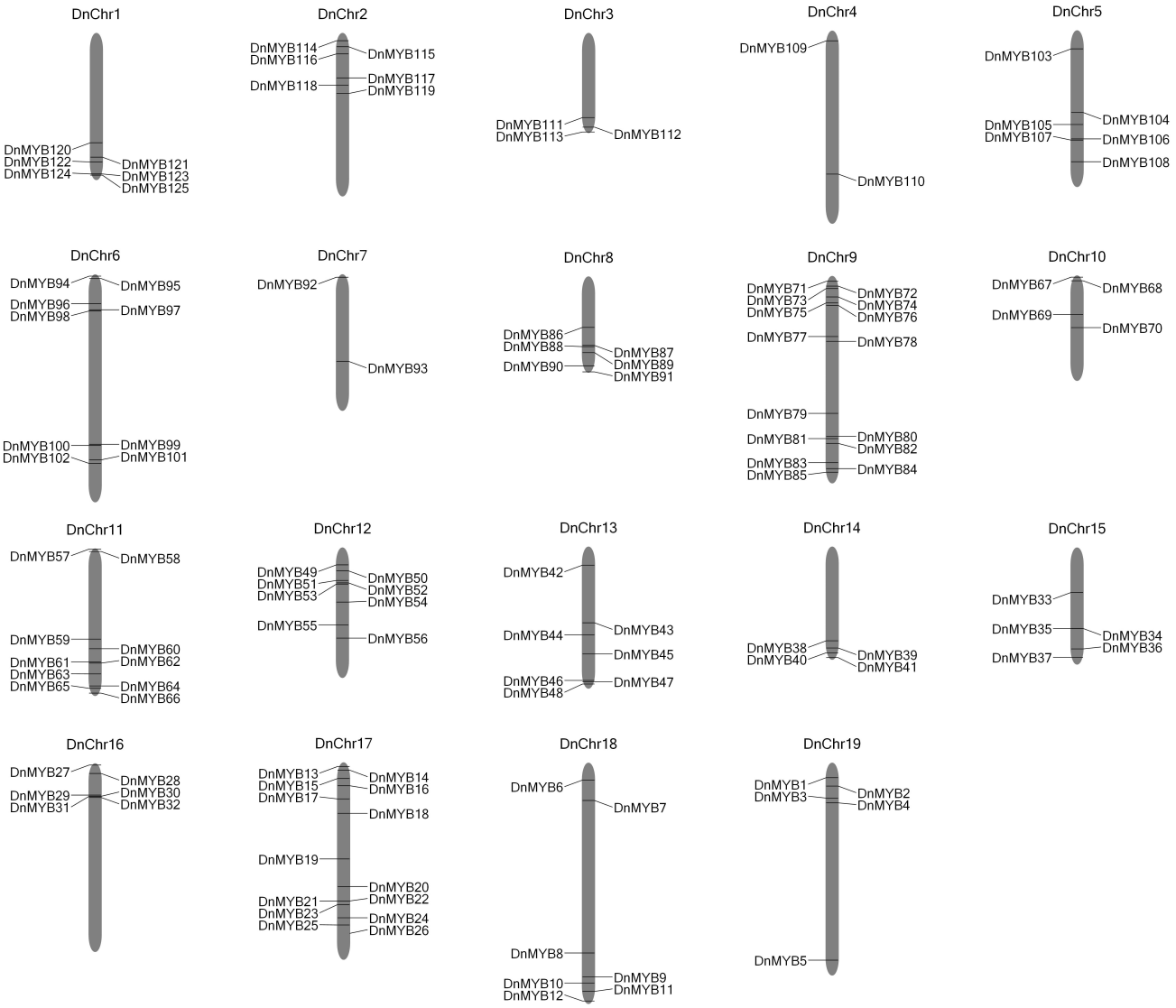

Fig. 3. Distribution of 125 *DnMYB* genes on chromosomes of *D. nobile*.

3.6 Prediction of Cis-Acting Elements in the Promoters of 125 DnMYB Genes

Cis-acting elements in promoters are bound by TFs and play an important role in the regulation of gene expression [33]. Here, we used the PlantCARE online prediction tool to identify *cis*-acting elements located within the first 2,000 bp upstream of the transcription start site of 125 *DnMYB* genes (**Supplementary Table 5**). Besides the basic elements, the predicted *cis*-acting elements could be divided into three groups, including hormone-responsive elements (ABA, salicylic acid, gibberellin, MeJA, auxin), abiotic stress elements (low temperature-responsive elements and drought-responsive elements), and metabolism-related elements (regulation of flavonoid biosynthesis) (**Supplementary Fig. 1**). The MeJA-responsive element was the most abundant element with 350 instances. It is well known that regulates a wide variety of physiological processes such as plant growth and development, especially the response of plants to biotic and abiotic stresses [34]. *DnMYB23* contained 24 MeJA-responsive elements in its 2-kb upstream regulatory region. The hormone-related elements identified included 210 ABA-responsive elements, 87 gibberellin-responsive elements, 64 auxin-responsive elements, and 58 salicylic acid-responsive elements. Among abiotic stress elements, there were 60 low temperature responsive elements, 3 of which were in the promoter region of *DnMYB100*. A total of 80 drought responsive elements were also predicted, with *DnMYB34*, *DnMYB37*, *DnMYB58*, *DnMYB6*, *DnMYB74*, and *DnMYB83* harboring 3. Finally, 14 response elements associated with the regulation flavonoid biosynthesis were also identified in some of the 2-kb sequences analyzed. These results suggested that the expression of *DnMYB* genes is controlled by complex regulatory networks.

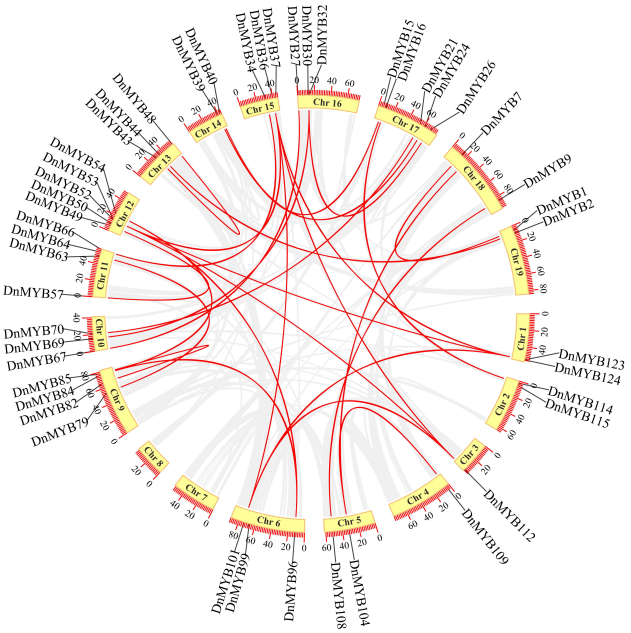

Fig. 4. Synteny analysis of interchromosomal relationships of *DnMYB* genes. Red lines indicate duplicated gene pair.

3.7 Expression Pattern of Candidate DnMYB Genes in Different Tissues

DnMYB genes in different subgroups may have different functions in the growth and development of *D. nobile*. To determine the spatial expression pattern of MYB genes in *D. nobile*, we measured the expression levels of 8 genes from eight different subtypes in four tissues (roots, stems, leaves, and flower) using RT-qPCR. *DnMYB8*, *DnMYB14*, *DnMYB26*, *DnMYB27*, *DnMYB81*, *DnMYB90*, *DnMYB108*, and *DnMYB116* belong to the S22, S2, S21, S1, S7, S4, S19, and S20 subfamilies, respectively. As shown in Fig. 6, 8 genes were expressed in all the tissues. The results indicated that *DnMYB8*, *DnMYB14*, *DnMYB26* and *DnMYB81* had similar expression patterns and were predominantly expressed in root. Meanwhile, the expression of *DnMYB27* was higher in flowers than in the other three tissues. Similarly, *DnMYB108* showed a tissue-specific expression pattern, showing 30-fold higher expression levels in flowers than in other tissues. *DnMYB90* was primarily expressed in the leaf and stem. *DnMYB116*, classified in the S20 subfamily, exhibited relatively high expression levels in the leaf and/or stem. The results suggested that these genes may play a role in the growth and development of *D. nobile*.

3.8 Expression Patterns of Candidate DnMYB Genes in Response to Biotic and Abiotic Stress

Based on the reported functions of R2R3-MYB in other plants, we selected 8 *DnMYBs* belonging to eight subfamilies to study the roles of R2R3-MYB in the regulation of secondary metabolism and the responses to abiotic stress for *D. nobile*. We measured the expression levels of these genes by RT-qPCR after treatment with ABA, MeJA and low-temperature (4 °C). Six of the 8 *DnMYB* genes-*DnMYB8*, *DnMYB14*, *DnMYB26*, *DnMYB27*, *DnMYB90*, and *DnMYB116*-showed increased expression levels at different times under the low temperature, ABA, and MeJA treatment (Fig. 7). While *DnMYB81* was the most responsive to low temperature, and showed reduced expression patterns under ABA- and MeJA- mediated stress. *DnMYB108* showed downregulated expression patterns under both low-temperature and MeJA stress. With ABA treatment, the expression of *DnMYB108* reached its lowest level at 12 h and peaked after 24 h.

4. Disscussion

4.1 Evolutionary Analysis of the DnMYB Gene Family

R2R3-MYB TFs have been identified in many plants, including *Arabidopsis* [32], maize [35], and soybean [30], etc. To further explain the evolutionary relationship of *DnMYB* genes, we performed a phylogenetic analysis based on 125 DnMYB proteins and 126 AtMYB proteins. Within the phylogenetic tree, these proteins were divided into 26 subgroups. Among them, S21 subgroup had the highest number of DnMYB proteins. In addition, we also found that some *DnMYB* genes had not been classified, indicating that their protein products may have special functions. We further analyzed the conserved motifs of the DnMYB proteins based on evolutionary relationships and identified a total of 10 conserved motifs. Most DnMYB proteins shared 3 motifs (motifs 1, 5, and 8; Fig. 2), which suggested that these motifs may have species specific functions. Our results indicated that although motif composition varied according to subgroup, the similarity in motif composition within the same subgroup was very high. The motif composition of closely related members in phylogenetic trees is consistent, and there have also been reports of tobacco [36]. Gene structure usually reflects the evolution of gene family [37]. In our gene structure analysis, we found that most genes in the same subgroup had either two or three exons, which was consistent with that reported for *D. officinale* and *P. aphrodite* [15]. These results indicate that the DnMYB protein is relatively conserved during evolution.

4.2 DnMYB Genes Play Essential Roles in D. nobile Growth and Response to Biotic and Abiotic Stresses

Low temperature, drought, and other stresses can seriously affect the growth and variety of *D. nobile*. Secondary metabolites such as flavonoids and anthocyanins contribute to plants adaptations to hostile environments. Meanwhile, flavonoid biosynthesis can be induced under a wide range of abiotic stresses. Accordingly, stress resistance of *D. nobile* can be enhanced via the regulation of genes related to secondary metabolite production. MYB genes contribute to the development of trichomes and root hairs, and play a crucial role in regulating the synthesis of plant secondary metabolites, such as flavonoids and antho-

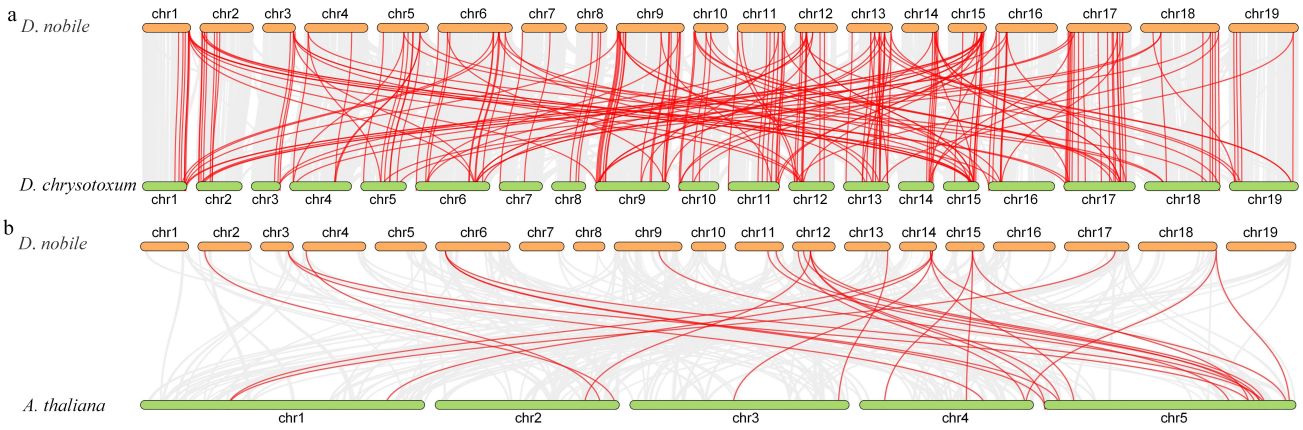

Fig. 5. Synteny analysis of *DnMYBs* genes in the genomes between *D. nobile* and *D. chrysotoxum* or *A. thaliana*. (a) *D. nobile* and *D. chrysotoxum*. (b) *D. nobile* and *A. thaliana*. The gray lines show collinear blocks. The red and blue lines indicate the syntenic gene pairs between *D. nobile* and *D. chrysotoxum* or *A. thaliana*, respectively.

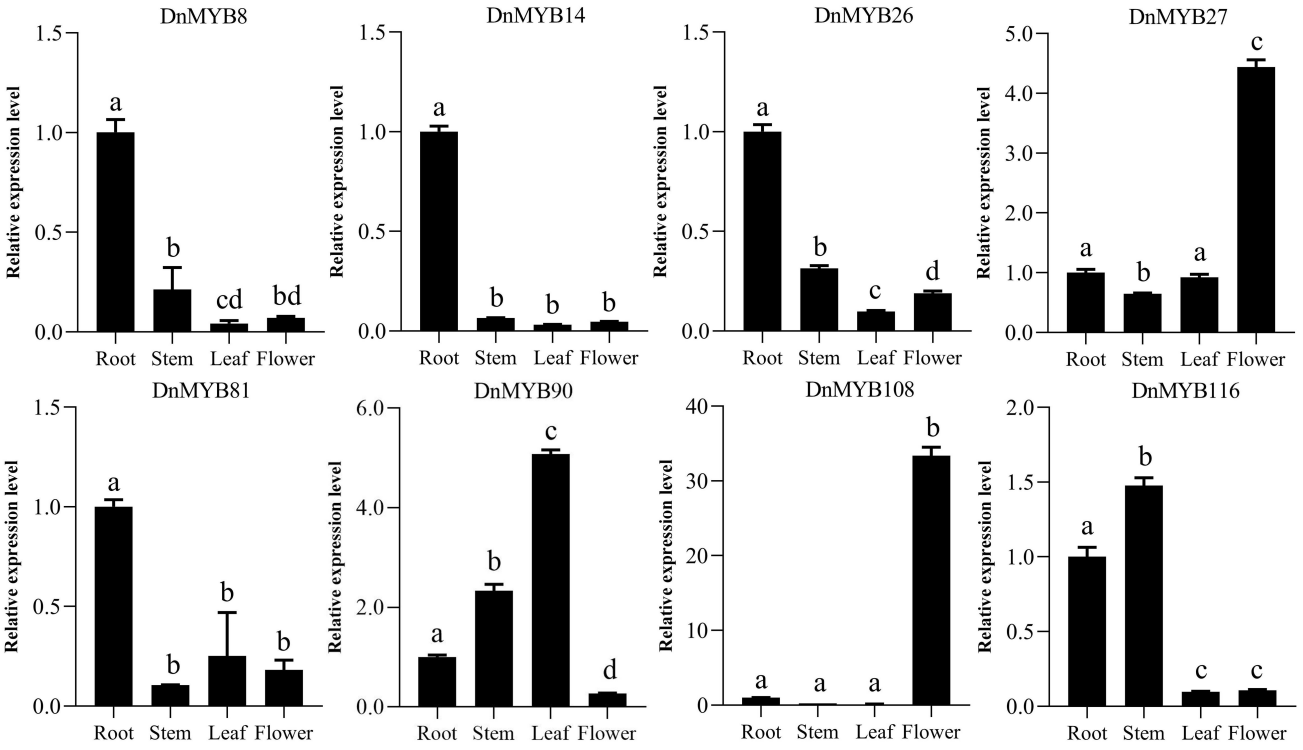

Fig. 6. Relative expression levels of 8 selected *DnMYB* genes in different tissues. Error bars indicate the standard error from three independent replicates. Different lowercase letters (a, b, c, and d) are significantly different (p-value < 0.05).

cyanins, that help plants resist stress [38]. To identify *DnMYB* genes that may be involved in regulating growth, development, and secondary metabolic synthesis of *D. nobile*, we selected 8 genes, each from a different subgroup, and measured their expression levels in different tissues and under different stresses. Gene functions are closely related to tissue-specific expression. Flavonoids, the major pigments in plants, have attracted substantial research attention owing to their important biological properties. It has been reported that flavonoid biosynthesis in plants can be induced under a wide range of abiotic stresses [38]. For instance, in wheat, the key enzymes in the flavonoid biosynthesis pathway were found to be upregulated by drought stress [39]. *AtMYB4* and *AtMYB7*, which belong to the S4 subfamily, are related to the flavonol synthesis pathway [40]. Similarly, the *AtMYB11*, *AtMYB12* and *AtMYB111* genes within the S7 subfamily are involved in the regulation of flavonol biosynthesis in *Arabidopsis* [41]. Genes with high homology in the same branch of a phylogenetic tree generally have high sequence similarity and may also have similar functions. The *DnMYB90* gene, which was also categorized into the S4 subfamily, is a homolog of *AtMYB4*

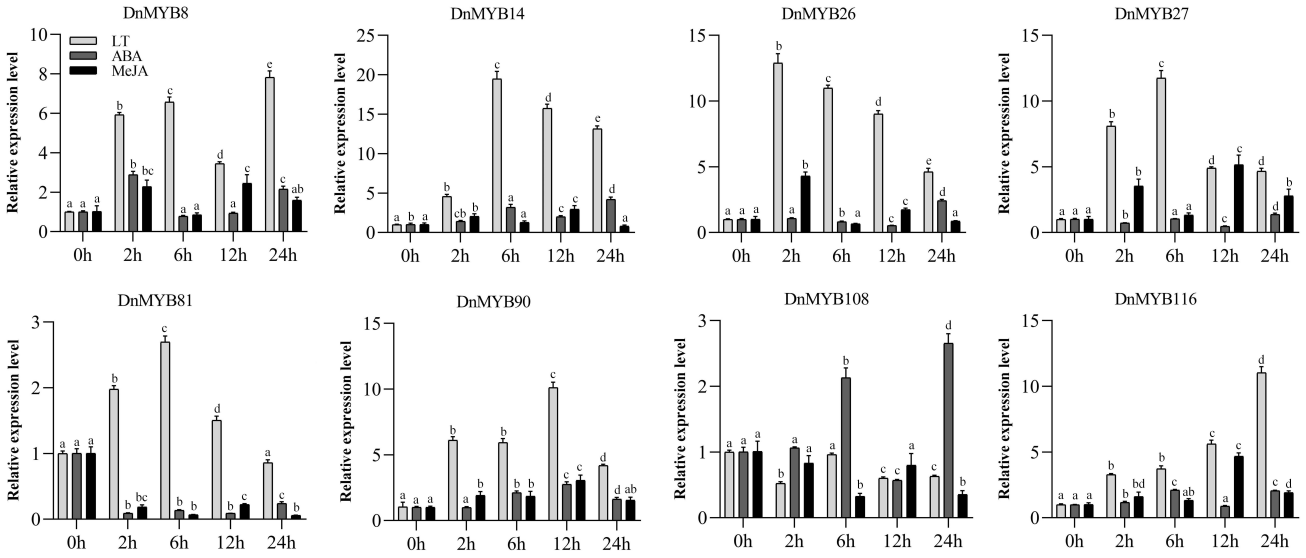

Fig. 7. Expression of 8 *DnMYB* genes at 0, 2, 6, 12, and 24 h after various stresses treatment. LT means Low temperature, ABA means abscisic acid, MeJA means methyl jasmonate. Error bars indicate the standard error from three independent replicates. Different lowercase letters (a, b, c, d and e) are significantly different (p-value < 0.05).

and *AtMYB7*. RT-qPCR analysis showed that although *DnMYB90* was expressed in all the tissues of *D. nobile*, the highest expression level was detected in the leaf. *DnMYB90* was also expressed at very high levels under phytohormonal and abiotic stresses (ABA, MeJA, low-temperature stress), but it was most responsive to low temperature. *DnMYB81*, belonging to the S7 subfamily, exhibited close homology with *AtMYB11*, *AtMYB12*, and *AtMYB111*. *DnMYB81*, belonging to the S7 subfamily, was highly homologous to *AtMYB11*, *AtMYB12*, and *AtMYB111*. The results of the RT-qPCR analysis showed that *DnMYB81* was expressed in all the tissues, but was most highly expressed in the root. *DnMYB81* was also responsive to phytohormonal and abiotic stresses. This gene was most responsive to low temperature and showed downregulated expression patterns under ABA and MeJA stress. Future, studies should focus on whether *DnMYB81* and *DnMYB90* can regulate flavonoid synthesis.

Several *R2R3-MYB* genes were reported to be involved in regulating responses to biotic and abiotic stresses. For example, the overexpression of *AtMYB94* and *AtMYB96*, the S1 subfamily genes, enhances drought tolerance in plants [42]. *AtMYB14*, a member of the S2 subfamily, regulates cold tolerance in *Arabidopsis* [43]. Under conditions of phosphate starvation, the S2 subfamily *AtMYB20*, which plays a role in plant responses to salt and drought stress, directly regulates the expression of miR399f [44]. Meanwhile, the Overexpression of the S21 subfamily member *AtMYB52* confers ABA hypersensitivity and drought tolerance [45]. *AtMYB44*, belonging to the S22 subfamily, responds to salt, MeJA and drought [46]. To identify *DnMYB* genes related to hormonal and abiotic stresses, we detected the expression levels of *DnMYB* genes from the S1, S2, S20, S21, and S22 subgroups following low temperature, ABA, and MeJA treatments. We found that *DnMYB27* in the S1 subfamily, *DnMYB14* in the S2 subfamily, *DnMYB116* in the S20 subfamily, *DnMYB26* in the S21 subfamily, and *DnMYB8* in the S22 subfamily, which we found were homologous to stress-response genes, were expressed in in roots, stems, leaves, and flowers. To further identify candidate genes involved in hormone signaling pathways or abiotic stress responses, we measured their expression levels under low temperature, ABA and MeJA stress. The results showed that the five genes showed increased expression levels at different times under different stress, which was consistent with the presence of the relevant *cis*-acting elements in their promoters (Figs. 2,7). Interestingly, the expression level of *DnMYB108*, classified in the S19 subfamily, gradually decreased after treatment with cold and MeJA stress. These results may suggest that members of this subfamily play an important role in the response of *D. nobile* to abiotic stress. Some of the *DnMYB* genes screened in this study may play a role in responses to abiotic stresses such as low temperature, ABA and MeJA stress; however, the underlying mechanism remains unclear and requires future clarification.

5. Conclusions

In this study, a total of 125 *DnMYB* genes were identified and subsequently subjected to phylogenetic, chromosome localization, gene duplication, conserved domain and gene structures analysis. These results are helpful to verify their evolutionary relationship with other plant species. In addition, the selected *DnMYB* gene is differentially expressed in different tissues and responds to low temperature, ABA, and MeJA, indicating that the *R2R3-MYB* gene plays a crucial role in the growth, development, and stress

response of *D. nobile*. These results provide useful information for further studies relating to the *R2R3-MYB* gene family.

Abbreviations

TFs, transcription factors; RT-qPCR, Reverse transcription-quantitative real-time PCR; ABA, abscisic acid; MeJA, methyl jasmonate; HMM, hidden Markov model; NCBI, National Center for Biotechnology Information; MW, molecular weight; pI, isoelectric point.

Availability of Data and Materials

All data generated or analyzed in this study are included in this article (Supplementary file). The genome sequences of *D. nobile*, *D. chrysotoxum* and *Arabidopsis* were downloaded from the https://www.ncbi.nlm.nih.gov/genome/?term=dendrobium+nobile, https://www.ncbi.nlm.nih.gov/genome/?term=Dendrobium+chrysotoxum, and https://www.arabidopsis.org/. Further inquiries should be directed to the corresponding authors.

Author Contributions

Conceptualization—LW; Methodology and writing original draft preparation—LW, JF, XS, DP; Writing—review and editing—DP, SX; Data curation—LW, SX. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Dendrobium nobile were provided by Xianglin He, ”Heir of D. huoshanense intangible cultural heritage”, Dendrobium industry association, Anhui Province, China.

Acknowledgment

We thank Shuai Liu from the University of Hawaii Cancer Center for helping to revise the manuscript. We also thank Zhikai Liang from the Department of Horticultural Science at University of Minnesota for helpful comments on an earlier version of the manuscript.

Funding

This work was supported by was supported by National Natural Science Foundation of China (Grant No. U19A2009), Key Natural Science Research Projects in Anhui Universities (No. 2022AH050461), Open Project of Provincial and Ministerial Scientific Research Platform, Fuyang Normal University (No. FSKFKT010D) and Anhui University Collaborative Innovation Project (Grant No. GXXT-2019-043).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2901001.

References

[1] Zhang X, Wang M, Zhang C, Liu Z, Zhou S. Clinical study of Dendrobium Nobile Lindl intervention on patients with metabolic syndrome. Medicine. 2021; 100: e24574.

[2] Krishnatreya DB, Baruah PM, Dowarah B, Bordoloi KS, Agarwal H, Agarwala N. Mining of miRNAs from EST data in Dendrobium nobile. Bioinformation. 2020; 16: 245–255.

[3] Zhong H, Kong W, Gong Z, Fang X, Deng X, Liu C, *et al*. Evolutionary Analyses Reveal Diverged Patterns of *SQUAMOSA* Promoter Binding Protein-Like (*SPL*) Gene Family in *Oryza* Genus. Frontiers in Plant Science. 2019; 10: 565.

[4] Cao Y, Han Y, Li D, Lin Y, Cai Y. MYB Transcription Factors in Chinese Pear (Pyrus bretschneideri Rehd.): Genome-Wide Identification, Classification, and Expression Profiling during Fruit Development. Frontiers in Plant Science. 2016; 7: 577.

[5] Feller A, Machemer K, Braun EL, Grotewold E. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. The Plant Journal. 2011; 66: 94–116.

[6] Xiang L, Jian D, Zhang F, Yang C, Bai G, Lan X, *et al*. The cold-induced transcription factor bHLH112 promotes artemisinin biosynthesis indirectly via ERF1 in Artemisia annua. Journal of Experimental Botany. 2019; 70: 4835–4848.

[7] Shen Q, Huang H, Xie L, Hao X, Kayani SI, Liu H, *et al*. Basic Helix-Loop-Helix Transcription Factors AabHLH2 and AabHLH3 Function Antagonistically With AaMYC2 and Are Negative Regulators in Artemisinin Biosynthesis. Frontiers in Plant Science. 2022; 13: 885622.

[8] Martin C, Paz-Ares J. MYB transcription factors in plants. Trends in Genetics. 1997; 13: 67–73.

[9] Wei Q, Chen R, Wei X, Liu Y, Zhao S, Yin X, *et al*. Genome-wide identification of R2R3-MYB family in wheat and functional characteristics of the abiotic stress responsive gene TaMYB344. BMC Genomics. 2020; 21: 792.

[10] Yao X, Meng F, Wu L, Guo X, Sun Z, Jiang W, *et al*. Genome-wide identification of R2R3-MYB family genes and gene response to stress in ginger. The Plant Genome. 2022; e20258.

[11] Tombuloglu H, Kekec G, Sakcali MS, Unver T. Transcriptome-wide identification of R2R3-MYB transcription factors in barley with their boron responsive expression analysis. Molecular Genetics and Genomics. 2013; 288: 141–155.

[12] Millard PS, Kragelund BB, Burow M. R2R3 MYB Transcription Factors - Functions outside the DNA-Binding Domain. Trends in Plant Science. 2019; 24: 934–946.

[13] Zhou Z, Wei X, Lan H. CgMYB1, an R2R3-MYB transcription factor, can alleviate abiotic stress in an annual halophyte Chenopodium glaucum. Plant Physiology and Biochemistry. 2023; 196: 484–496.

[14] Zhou L, Yarra R, Yang Y, Liu Y, Yang M, Cao H. The oil palm R2R3-MYB subfamily genes EgMYB111 and EgMYB157 improve multiple abiotic stress tolerance in transgenic Arabidopsis plants. Plant Cell Reports. 2022; 41: 377–393.

[15] Fan H, Cui M, Li N, Li X, Liang Y, Liu L, *et al*. Genome-wide identification and expression analyses of R2R3-MYB transcription factor genes from two Orchid species. PeerJ. 2020; 8: e9781.

[16] Zhang T, Cui Z, Li Y, Kang Y, Song X, Wang J, *et al*. Genome-Wide Identification and Expression Analysis of MYB Transcription Factor Superfamily in *Dendrobium catenatum*. Frontiers in Genetics. 2021; 12: 714696.

[17] Liu S, Zhong H, Wang Q, Liu C, Li T, Peng Z, *et al*. Global Analysis of UDP Glucose Pyrophosphorylase (UDPGP) Gene Family in Plants: Conserved Evolution Involved in Cell Death. Frontiers in Plant Science. 2021; 12: 681719.
[18] Zhong H, Zhang H, Guo R, Wang Q, Huang X, Liao J, *et al*. Characterization and Functional Divergence of a Novel *DUF668* Gene Family in Rice Based on Comprehensive Expression Patterns. Genes. 2019; 10: 980.
[19] Deng X, An B, Zhong H, Yang J, Kong W, Li Y. A Novel Insight into Functional Divergence of the MST Gene Family in Rice Based on Comprehensive Expression Patterns. Genes. 2019; 10: 239.
[20] Kong W, Gong Z, Zhong H, Zhang Y, Zhao G, Gautam M, *et al*. Expansion and Evolutionary Patterns of Glycosyltransferase Family 8 in Gramineae Crop Genomes and Their Expression under Salt and Cold Stresses in *Oryza sativa* ssp. *japonica*. Biomolecules. 2019; 9: 188.
[21] Xu M, Wang Z, Zhang H, Pantazis D, Wang H, Li Q. A new Graph Gaussian embedding method for analyzing the effects of cognitive training. PLoS Computational Biology. 2020; 16: e1008186.
[22] Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, *et al*. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. Molecular Plant. 2020; 13: 1194–1202.
[23] Wang Z, Jiang W, Liu Y, Meng X, Su X, Cao M, *et al*. Putative genes in alkaloid biosynthesis identified in Dendrobium officinale by correlating the contents of major bioactive metabolites with genes expression between Protocorm-like bodies and leaves. BMC Genomics. 2021; 22: 579.
[24] Kong W, Zhong H, Deng X, Gautam M, Gong Z, Zhang Y, *et al*. Evolutionary Analysis of *GH3* Genes in Six *Oryza* Species/Subspecies and Their Expression under Salinity Stress in *Oryza sativa* ssp. *japonica*. Plants. 2019; 8: 30.
[25] Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L, Zhaofeng G, *et al*. The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Molecular Biology. 2006; 60: 107–124.
[26] Rushton PJ, Somssich IE, Ringler P, Shen QJ. WRKY transcription factors. Trends in Plant Science. 2010; 15: 247–258.
[27] Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L. MYB transcription factors in Arabidopsis. Trends in Plant Science. 2010; 15: 573–581.
[28] Yang L, Liu H, Hao Z, Zong Y, Xia H, Shen Y, *et al*. Genome-Wide Identification and Expression Analysis of *R2R3-MYB* Family Genes Associated with Petal Pigment Synthesis in *Liriodendron*. International Journal of Molecular Sciences. 2021; 22: 11291.
[29] Premathilake AT, Ni J, Bai S, Tao R, Ahmad M, Teng Y. R2R3-MYB transcription factor PpMYB17 positively regulates flavonoid biosynthesis in pear fruit. Planta. 2020; 252: 59.
[30] Du H, Yang SS, Liang Z, Feng BR, Liu L, Huang YB, *et al*. Genome-wide analysis of the MYB transcription factor superfamily in soybean. BMC Plant Biology. 2012; 12: 106.
[31] Liu X, Yu W, Zhang X, Wang G, Cao F, Cheng H. Identification and expression analysis under abiotic stress of the *R2R3-MYB* genes in *Ginkgo biloba* L. Physiology and Molecular Biology of Plants. 2017; 23: 503–516.
[32] Stracke R, Werber M, Weisshaar B. The R2R3-MYB gene family in Arabidopsis thaliana. Current Opinion in Plant Biology. 2001; 4: 447–456.
[33] Mikhaylichenko O, Bondarenko V, Harnett D, Schor IE, Males M, Viales RR, *et al*. The degree of enhancer or promoter activity is reflected by the levels and directionality of eRNA transcription. Genes & Development. 2018; 32: 42–57.
[34] Gomi K. Jasmonic Acid Pathway in Plants 2.0. International Journal of Molecular Sciences. 2021; 22: 3506.
[35] Du H, Feng BR, Yang SS, Huang YB, Tang YX. The R2R3-MYB transcription factor gene family in maize. PLoS ONE. 2012; 7: e37463.
[36] Yang J, Zhang B, Gu G, Yuan J, Shen S, Jin L, *et al*. Genome-wide identification and expression analysis of the R2R3-MYB gene family in tobacco (Nicotiana tabacum L.). BMC Genomics. 2022; 23: 432.
[37] Yang W, Feng L, Luo J, Zhang H, Jiang F, He Y, *et al*. Genome-Wide Identification and Characterization of *R2R3-MYB* Provide Insight into Anthocyanin Biosynthesis Regulation Mechanism of *Ananas comosus* var. *bracteatus*. International Journal of Molecular Sciences. 2023; 24: 3133.
[38] Pratyusha DS, Sarada DVL. MYB transcription factors-master regulators of phenylpropanoid biosynthesis and diverse developmental and stress responses. Plant Cell Reports. 2022; 41: 2245–2260.
[39] Colebrook EH, Thomas SG, Phillips AL, Hedden P. The role of gibberellin signalling in plant responses to abiotic stress. The Journal of Experimental Biology. 2014; 217: 67–75.
[40] Fornalé S, Lopez E, Salazar-Henao JE, Fernández-Nohales P, Rigau J, Caparros-Ruiz D. AtMYB7, a new player in the regulation of UV-sunscreens in Arabidopsis thaliana. Plant & Cell Physiology. 2014; 55: 507–516.
[41] Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K, *et al*. Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. The Plant Journal. 2007; 50: 660–677.
[42] Lee SB, Kim HU, Suh MC. MYB94 and MYB96 Additively Activate Cuticular Wax Biosynthesis in Arabidopsis. Plant & Cell Physiology. 2016; 57: 2300–2311.
[43] Chen Y, Chen Z, Kang J, Kang D, Gu H, Qin G. *AtMYB14* Regulates Cold Tolerance in *Arabidopsis*. Plant Molecular Biology Reporter. 2013; 31: 87–97.
[44] Baek D, Chun HJ, Kang S, Shin G, Park SJ, Hong H, *et al*. A Role for Arabidopsis miR399f in Salt, Drought, and ABA Signaling. Molecules and Cells. 2016; 39: 111–118.
[45] Park MY, Kang JY, Kim SY. Overexpression of AtMYB52 confers ABA hypersensitivity and drought tolerance. Molecules and Cells. 2011; 31: 447–454.
[46] Jung C, Seo JS, Han SW, Koo YJ, Kim CH, Song SI, *et al*. Overexpression of AtMYB44 enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis. Plant Physiology. 2008; 146: 623–635.