Identification and expression analysis under abiotic stress of the *R2R3-MYB* genes in *Ginkgo biloba* L.

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Abstract The R2R3-MYB gene family is the largest MYB subfamily in plants and is involved in the regulation of plant secondary metabolism and specific morphogenesis, as well as the response to biotic and abiotic stress. However, a systematic identification and characterization of this gene family has not been carried out in *Ginkgo biloba*. In this study, we performed a transcriptome-wide survey from four tissues of *G. biloba* to determine the genetic variation and expression pattern of the *R2R3-MYB* genes. We analyzed 45 *GbMYBs* and identified 42 with a complete coding sequence via conserved motif searches. The MYB domain and other motifs in *GbMYBs* are highly conserved with *Arabidopsis thaliana* AtMYBs. Phylogenetic analysis of the *GbMYBs* and AtMYBs categorized the R2R3-MYBs into 26 subgroups, of which 11 subgroups included proteins from both *G. biloba* and *Arabidopsis*, and 1 subgroup was specific to *G. biloba*. Moreover, the *GbMYBs* expression patterns were analyzed in different tissues and abiotic

stress conditions. The results revealed that *GbMYBs* were differentially expressed in various tissues and following abiotic stresses and phytohormone treatments, indicating their possible roles in biological processes and abiotic stress tolerance and adaptation. Our study demonstrated the functional diversity of the *GbMYBs* and will provide a foundation for future research into their biological and molecular functions.

Keywords Transcriptome · R2R3-MYB transcription factors · *Ginkgo biloba* L. · Stress response · Phytohormone

Introduction

Plants play important role in the rise of sedentary human civilization. An important aspect of cultivation of plants for food, fiber, biofuel, medicine and other products is to sustain and enhance human life (Hricova et al. 2016; Sakar et al. 2016; Saridas et al. 2016; Solmaz et al. 2016). Transcription factors (TFs) play key role in regulating plant development and stress responses. The functions of plant R2R3-MYB transcription factors have been characterized and were associated with the control of plant-specific processes, including primary and secondary metabolism, developmental processes, cell fate and identity, and stress responses (Dubos et al. 2010).

MYB proteins constitute one of the largest transcription factor families in eukaryotes and are characterized by a highly conserved DNA-binding domain (DBD) known as the MYB domain (Rabinowicz et al. 1999). The MYB domain contains up to four imperfect repeats, referred to as R1, R2, R3, and R4. Each repeat contains approximately 52 amino acid residues and encodes three α -helices, with the second and third helices forming a helix-turn-helix (HTH)

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structure when bound to specific promoter sequences (Ogata et al. 1992; Stracke et al. 2001). The third α -helix of each repeat is a DNA-recognition helix that binds specific DNA sequences in the major groove (Ogata et al. 1994; Ogata et al. 1996). Typically, three regularly spaced tryptophan (W) residues, which play a significant role in sequence-specific DNA binding, characterize the MYB repeat and form a hydrophobic cluster in the three-dimensional HTH structure (Ogata et al. 1995; Wilkins et al. 2009). In plants, the first Trp of R3 is always substituted by Phe or Ile (Ambawat et al. 2013). This alteration in R3 may lead to functional variation in specific cell processes.

In plants, the MYB family is classified into four subfamilies, 1R-, R2R3-, R1R2R3- and 4R-MYBs based on the number of DBD repeats (Dubos et al. 2010). Among the four MYB subfamilies, R2R3-MYB proteins have two adjacent DBD repeats, known as R2 and R3, in the N-terminal and comprise the largest MYB subfamily in plants (Goral et al. 2012). The length of this highly conserved domain is approximately 105 amino acids, and it is critical for the function and DNA binding (Wu et al. 2003b). In addition to the MYB domain in the N-terminal, R2R3-MYB proteins have other conserved motifs, such as activation or repression domains in the C-terminal (Jin and Martin 1999). Compared to the N-terminal, the C-terminal domains are not notably conserved and are highly divergent. They always function as trans-acting domains (TAD) responsible for the regulatory activity of the protein (Ambawat et al. 2013). Based on the conservation of C-terminal motifs, the large R2R3-MYB family in *Arabidopsis thaliana* has been divided into 22 subgroups, which often categorize proteins based on their functional relationships (Kranz et al. 1998; Stracke et al. 2001). This classification was also applied to R2R3-MYB proteins in other plant species, although the groups of several species diverge slightly (Du et al. 2012; Li and Lu 2014; Stracke et al. 2014; Tombuloglu et al. 2013; Wang et al. 2015).

The first plant MYB gene, *COLORED1*, was isolated from *Zea mays* and encodes a c-MYB-like TF involved in anthocyanin biosynthesis (Paz-Ares et al. 1987). Subsequently, a large number of plant MYB TFs have been investigated in numerous plants. To date, 8746 MYB sequences are available in the plant TF database (<http://plantfdb.cbi.pku.edu.cn/>). Large amounts of data on the role of MYB proteins in plants have provided comprehensive descriptions and clear classifications of plant MYBs. Based on the sequences of MYB domains from several organisms, evolutionary studies proposed that plant R2R3-MYB genes evolved from an R1R2R3-MYB gene ancestor and that the first repeat was lost (Lipsick 1996; Rosinski and Atchley 1998). Additionally, the R1R2R3- and R2R3-MYB genes coexisted in eukaryotes before the divergence of plants and animals. In MYB evolution in

plants, the expansion of the R2R3-MYB family was considerable and believed to be correlated with its diverse functions (Riechmann et al. 2000).

In recent years, as a growing number of plant genomes have been sequenced, the R2R3-MYB families have been annotated genome-wide in several plant species. A different number of gene family members have been reported in various species. For example, *A. thaliana* has 126 members, *Oryza sativa* has 109 members, *Populus trichocarpa* has 192 members, *Salvia miltiorrhiza* has 110 members, *Beta vulgaris* has 70 members, *Gossypium raimondii* has 205 members, *Z. mays* has 157 members, and the *Brassica rapa* ssp. *Pekinensis* has 256 members (Du et al. 2012; Dubos et al. 2010; He et al. 2016; Li and Lu 2014; Stracke et al. 2014; Wang et al. 2015). To date, the *B. rapa* ssp. *Pekinensis* has the largest R2R3-MYB family among the sequenced plants. The variation in the number of R2R3-MYB family members from different plant species may be due to genomic duplication events or environmental adaptation, indicating the specificity after divergence from the last common ancestor (Cannon et al. 2004; Flagel and Wendel 2009; Wilkins et al. 2009).

Genome-wide identification of R2R3-MYB families allows for an in-depth elucidation of their characteristics and regulatory mechanisms in plants. The role of the R2R3-MYB family in the model plant *Arabidopsis* has been extensively studied. Comparative phylogenetic studies of R2R3-MYB proteins from *Arabidopsis* and other plant species suggested that the function of R2R3-MYBs in the same subgroup was usually consistent with that of *Arabidopsis* (Li and Lu 2014). Thus, phylogenetic-based analysis may be used to predict the function of MYBs from other organisms. Moreover, application of de novo sequencing also provides another method to investigate the transcripts of the R2R3-MYB gene family when there is a lack of genome sequencing data in plants (Zhang et al. 2016).

Ginkgo biloba L. is the only remaining species of Ginkgoaceae and is often called a “living fossil” (Shen et al. 2006). Geological records have shown that *G. biloba* exhibits minor changes in its morphology, indicating its strong adaptability in changing environments and tolerance of harsh conditions (Royer et al. 2003). As a dioecious plant, *G. biloba* is one of the most popular functional plants used worldwide today, especially for medicinal purposes (Cheng et al. 2013). Among extracts of the *G. biloba* leaves, EGb 761 is one of the most widely used preparations and contains active compounds such as 24% ginkgo flavonoid glycosides and 6% terpene trilactones (DeFeudis and Drieu 2000). Flavonoids have remarkable pharmacological and clinical effects and are widely used in many countries to treat cardiovascular diseases and cancer (DeFeudis et al. 2003). Flavonoid biosynthesis is derived from the phenylpropanoid pathway, and their structural

genes are regulated by various TFs, including HLHs, MYBs and W40 proteins (Koes et al. 2005). In *G. biloba*, most structural genes of the flavonoid biosynthetic pathway have been isolated, including *PAL*, *CHS*, *CHI*, *F3H*, *FLS*, and *ANS* (Xu et al. 2012). Additionally, MYB-binding sites were found in the promoter regions of several ginkgo flavonoid biosynthetic genes, such as *GbCHS*, *GbFLS*, and *GbCHI* (Cheng et al. 2011; Li et al. 2014; Xu et al. 2012). However, knowledge of the function of ginkgo *R2R3-MYB* genes is limited because very few reports have examined ginkgo MYBs. In this study, to evaluate the role of MYB genes in ginkgo development, defense and flavonoid biosynthesis, we performed a transcriptome-wide analysis of the *R2R3-MYB* gene subfamily in *G. biloba*.

Materials and methods

Identification of R2R3-MYB transcriptional factors in ginkgo

With Illumina pair-end sequencing technology, the transcriptome of *G. biloba* was previously sequenced using four *G. biloba* organs, including leaves of male trees, leaves of female trees, stamens, and gynoecia, which yielded 67,254, 59,527, 30,771 and 35,113 unigenes, respectively (unpublished data). All unigenes were annotated using BLAST alignments with query databases, including NCBI-Nr, KEGG, Swiss-Prot, and COG. These transcriptomes provided datasets for gene screening and transcriptional profiling in *G. biloba*, for which the genome is not sequenced. For identifying the R2R3-MYB TF proteins from the transcriptomic data, the key words “MYB” and “R2R3 MYB” were used as queries to search against these annotated unigenes. The 5' terminal fragments of the encoded R2R3-MYB genes were obtained from the search results. When *R2R3-MYB* genes identified from the transcriptome overlapped completely, the gene with the longer nucleotide sequence was reserved. The coding sequences were manually retrieved with ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) and were then validated by BLASTX.

Analysis of the MYB domain and other motifs

For the analysis of MYB domains of *G. biloba* R2R3-MYB TF proteins, the amino acid sequences of R2 and R3 repeats were aligned by ClustalW using MEGA version 5.1 software. The WebLogo server (<http://weblogo.berkeley.edu/logo.cgi>) was used to create the sequence logos for R2 and R3 MYB repeats by submitting the multiple alignment sequences. The MEME Suite v4.11.1 was used to predict

potential protein motifs outside the MYB domain (Bailey et al. 2009). The parameters of the MEME suite were based on Li and Lu (2014). Only motifs with an e-value <1e-10 were collected for further analysis.

Phylogenetic analysis of R2R3-MYB proteins

Multiple sequence alignment, phylogenetic, and molecular evolutionary analyses were conducted by MEGA v5.1 using the amino acid sequences of R2R3-MYBs. A neighbor-joining tree was constructed with the following parameters: Poisson correction, complete deletion and bootstrap analysis with 1000 replicates. For a better analysis of the subgroups of the putative ginkgo R2R3-MYB family, 125 *Arabidopsis* R2R3-MYB proteins were included in the phylogenetic analysis. *Arabidopsis* R2R3-MYB proteins were obtained from Stracke et al. (2001), and their amino acid sequences were downloaded from the Arabidopsis Information Resource (TAIR, <https://www.arabidopsis.org/>). A known ginkgo R2R3-MYB TF was obtained from Xu et al. (2014) and was renamed GbMYB0. Sequences of the 43 GbR2R3-MYBs and 125 AtR2R3-MYBs used for tree building are listed in Table S1.

Plant materials and stress treatments

Eighteen-year-old ginkgo trees were grown in the outdoor ginkgo garden of Nanjing Forestry University in Jiangsu Province, China. For tissue-specific expression analysis, leaves, stems, roots, stamens and gynoecia were collected from the 18-year-old seedlings. After collection, three biological replicates of each tissue were immediately frozen in liquid nitrogen and then stored separately at −80 °C prior to total RNA extraction.

To maintain consistency in the stress test, we used suspended cells as materials in the abiotic stresses and hormonal treatments. Mature zygotic embryos of *G. biloba* were incubated to initiate the callus cultures in the MS induction medium, which was supplemented with 2 mg L^{−1} 6-benzyladenine (6-BA) and 1.5 mg L^{−1} naphthaleneacetic acid (NAA). The calluses were transferred to MS liquid medium supplemented with 6-BA (2 mg L^{−1}) and NAA (1.5 mg L^{−1}) after two subcultures and were then cultured on a rotary shaker at 100 rpm, in the light at 25 ± 1 °C. The suspension cultures were subcultured three times at 1 week intervals, and the calluses that appeared pale yellow-white, translucent, or browning were omitted. The chilling and heat treatments were performed by placing the callus lines in a 4 or 40 °C growth room, respectively. For the investigation of inducible elicitors, the callus lines were treated with 100 μmol L^{−1} SA, 100 μmol L^{−1}

ABA, 100 $\mu\text{mol L}^{-1}$ MeJa, 40 $\mu\text{mol L}^{-1}$ ET separately and 200 mmol L^{-1} sodium chloride (NaCl) (Gong et al. 2006; Liao et al. 2015; Novo-Uzal et al. 2014). The calluses without any treatment were used as controls, and samples were collected at 3, 6, 12, 24 and 48 h after the treatments.

RNA extraction and qRT-PCR analysis

The total RNA was extracted with TRIzol (Invitrogen, US) according to the manufacturer's instructions and was then treated with RNase-free DNase I (Fermentas, Germany) at 37 °C for 30 min. The quantity and quality of RNA samples were assessed by agarose gel electrophoresis and a Nanodrop ND-2000 (Nanodrop Technologies, US). Reverse transcription-polymerase chain reaction was performed using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany).

qRT-PCR analysis was performed to investigate the transcription profile of selected genes in different subgroups. qRT-PCR was carried out on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Roche, Germany). The glyceraldehyde-3-phosphate dehydrogenase gene (*GbGAPDH*, GenBank Accession no. L26924) was used as the reference gene (Xu et al. 2008). The primers for selected genes and *GbGAPDH* were designed and are listed in Table 1. Real-time PCR was performed in a reaction volume of 20 μl containing 50 ng template cDNA, 2 $\times$ SYBR Green Real-time PCR Master Mix and 2.5 mM of each primer. The following qPCR conditions recommended by the manufacturer were used: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Three biological replicates were run for each gene at different tissues and stress treatment conditions. qRT-PCR data were calibrated relative to the corresponding gene expression level at time zero for each treatment, following the $2^{-\Delta\Delta\text{CT}}$ method for relative quantification (Livak and Schmittgen 2001).

Statistical analysis

Statistical analyses were performed by one-way analyses of variance (ANOVA), and mean differences were compared by lowest standard deviation (LSD) test using SPSS 17.0 software. $P < 0.05$ was considered to be statistically significant. All data are shown as the mean $\pm$ standard error of the mean.

Results

Identification of *GbR2R3-MYB* genes in ginkgo

Based on the data obtained from the ginkgo transcriptome assembly, 83 unigenes annotated as R2R3-MYB TFs were screened. After filtering the completely overlapping sequences, we analyzed all annotated putative R2R3-MYB sequences using open reading frame (ORF) prediction and then validated the results by BLASTP alignment with the DBDs of MYBs. Only sequences containing the two MYB domains were considered members of *R2R3-MYB* gene family. Then, ClustalW was used to align the amino acid sequences of the MYB repeats and validate the consensus R2 and R3 repeats. Finally, a total of 45 putative *GbMYBs* were identified as R2R3-MYB candidate genes in the ginkgo transcriptomes, and 42 contained complete coding regions (Table S1 and Table S2). The 42 genes with full-length ORFs were named *GbMYB1* to *GbMYB42*, and the remaining three *GbMYBs* were named *GbMYB43* to *GbMYB45*.

MYB domain of the *G. biloba* R2R3-MYB family

To elucidate the MYB-specific characteristics, we performed a multiple sequence alignment, and sequence logos of the R2R3-MYBs were generated for ginkgo and *Ara-bidopsis* (Fig S1 and Fig. 1). The results showed that the MYB domain included two DBD repeats that are highly conserved in the N-terminal, with a length of

Table 1 Primers for qRT-PCR

Gene	Primer forward sequence (5'–3')	Primer reverse sequence (5'–3')	Product size (bp)
<i>GAPDH</i>	GGTGCCAAAAAGGTGGTCAT	CAACAACGAACATGGGAGCAT	58
<i>GbMYB5</i>	CTCTGGAGTGCTGGATTGGATG	CAAGTATGGAAGCCAATCTCTGC	133
<i>GbMYB8</i>	GTTGAGACCTCTGCGAATGCTG	TGGGAGCGAGAGAGGATTGAA	103
<i>GbMYB14</i>	AGGCACCGCTGAAGGACAA	TGGAGTAGGGTGGCTTTGATAGATA	110
<i>GbMYB15</i>	ATGTCCGCTGTCCTCAGATTCA	TCAATGGCACGGTTGTTCTCA	180
<i>GbMYB26</i>	AATGCCCGTGAAGAACCTCC	CGTTGGTATTCACTCCATTGCC	97
<i>GbMYB30</i>	TCCCTGAACCACACGCTACC	AACCGCTACTTCTGGCTGCTC	77
<i>GbMYB31</i>	ACAATCTTCCCTTGATGACGACG	TCAACGCTGCTCGGAATGG	103
<i>GbMYB42</i>	TCACGAGGCACAAACAGCG	CCCAGAAGCCAGAGTGGATTG	117

Fig. 1 Comparison of R2 and R3 sequences in R2R3-MYB TFs from *G. biloba* and *Arabidopsis*. R2 repeats in MYBs from *G. biloba* (a) and *Arabidopsis* (b). R3 repeats in MYBs from *G. biloba* (c) and *Arabidopsis* (d). The positions of the three α -helices forming the R2

and R3 repeats are labeled Helix 1 to Helix 3. Highly conserved Trp (W) and Phe (F) residues are labeled with asterisks, and the differing residues between *G. biloba* and *Arabidopsis* are labeled with triangles

approximately 104 amino acids. The R2 and R3 residues of the *G. biloba* R2R3-MYBs were highly consistent with those in *Arabidopsis*. In both ginkgo and *Arabidopsis*, the R2 repeat has three highly conserved Trp (W) residues at positions 6, 26, and 46. Similarly, the R3 repeat contains three highly conserved residues, including a Phe (F) and two Trp residues at positions 59, 78, and 97. A significant proportion of these 43 GbMYBs (33%) had a substitution of Ile (I) at position 59; two GbMYBs (GbMYB0 and GbMYB5) had a substitution of Leu (L), and GbMYB13 had a substitution of Val (V).

In addition to these highly conserved Trp/Phe residues, there were also 16 residues that were completely conserved in the MYB domain of GbMYB proteins (Fig S1). These residues were predominantly distributed between the last two conserved Trp residues of the R2 and R3 repeats and were particularly concentrated in the third helix. Less conserved residues were found between the first two conserved Trp residues (Fig. 1). Due to its DNA-recognition function, helix3 of the repeats, which directly contacts the DNA, is more conserved than helix1 and helix2. A bHLH-binding consensus motif, D/E-L-x2-R/K-x3-L-x6-L-x3-R, was found from Asp/Glu65 to Arg84 in the R3 domain of 10 GbMYBs (*GbMYB1*, -3, -10, -21, -23, -31, -36, -39, -41, and -45) and interacts with R-Like bHLH proteins (Fig S1). A highly conserved motif, LRPD, is positioned from Leu50 to Asp53 and was found in the linker region of R2 and R3 in ginkgo and *Arabidopsis*. Additionally, this motif was also observed in *P. trichocarpa*, *Z. mays* and *S. miltiorrhiza* (Du et al. 2012; Li and Lu 2014; Wilkins et al. 2009). Six residues of the R2 repeat and four residues of the R3 repeats differed between ginkgo and *Arabidopsis* (Fig. 1).

Conserved motifs outside the MYB domain

In addition to the MYB domain, the R2R3-MYB proteins contain many other important functional motifs. These motifs are less conserved than the MYB domain but are often associated with important functions. MEME suite was used to analyze these motifs, and 27 putative motifs were identified outside of the MYB domain of ginkgo and *Arabidopsis* R2R3-MYBs (Fig S2). The motifs ranged from 7 to 120 amino acids, and 5 motifs were specific to GbMYB. No motifs were identified in 16 AtMYBs and 4 GbMYBs (*GbMYB27*, -29, -34, -36). Among these motifs, motifs 4 and 5 were the two most common motifs and were positioned downstream and upstream of the MYB domain, respectively. Eleven motifs were found in both ginkgo and *Arabidopsis*, suggesting that the motifs of R2R3-MYBs were evolutionarily conserved (Fig S2). Although these motifs are functionally important, most of them are novel and should be characterized in divergent species.

Phylogenetic analysis of R2R3-MYB proteins in *G. biloba* and *Arabidopsis*

To examine the relationship between *AtR2R3MYBs* and these putative *GbR2R3MYBs*, a phylogenetic tree was constructed using the full-length R2R3-MYB amino acid sequences (Fig. 2). As shown in Fig. 2, there was a close resemblance between many R2R3-MYBs from *G. biloba* and their counterparts in *Arabidopsis*. Based on the phylogenetic tree and the classification model of *Arabidopsis* that was constructed with specific sequence motifs at the C-terminal ends, R2R3-MYBs in ginkgo and *Arabidopsis* were classified into 26 subgroups (S1–S26). R2R3-MYBs in a subgroup shared at least one motif outside of the MYB domain. Of these subgroups, S8, S17 and S26 were novel, while the others had been identified in previous studies of *Arabidopsis* (Dubos et al. 2010; Stracke et al. 2001). Among the 26 subgroups, 11 subgroups included proteins from both ginkgo and *Arabidopsis* (S4, S7, S8, S9, S11, S13, S17, S18, S21, S22, S23), and 1 subgroup was specific to ginkgo (S26). As the MYB proteins in the same subgroups usually control the same metabolic pathways, our results will provide further support for functional prediction of these *GbMYB* genes. To predict the biological functions of ginkgo MYBs, we selected 8 *GbMYB* genes (*GbMYB5*, -8, -14, -15, -26, -30, -31, -42) from different subgroup as candidate genes because the functions of their *Arabidopsis* counterparts have been well studied.

Expression patterns of candidate *GbR2R3-MYBs* in different tissues

To investigate the expression pattern of *GbMYBs* in different ginkgo tissues, we performed qRT-PCR analysis using the primers shown in Table 1. The results indicated that *GbMYB8* and *GbMYB14* shared similar expression patterns and were predominantly expressed in gynoecia, while *GbMYB26* was predominant in the leaves (Fig. 3). *GbMYB42* was principally expressed in stamens, and *GbMYB15* showed strong expression in reproductive organs (stamens and gynoecia). *GbMYB31* was predominantly expressed in the leaves and gynoecia, and the other 2 MYBs (*GbMYB5* and *GbMYB30*) had high expression in at least three of the tissues. The results suggested that these genes have a possible role in the growth and development of *G. biloba*.

Expression patterns of *GbMYBs* following abiotic stresses and phytohormone treatment

To determine if the *GbMYBs* were involved in the response to abiotic stresses and phytohormones, we measured the

Fig. 2 Comparative phylogenetic analysis of ginkgo R2R3-MYB TFs with those of *Arabidopsis*. The tree was constructed using the neighbor-joining method with a Poisson correction model and 1000 bootstrap replicates. The tree was divided into 26 main subgroups,

and proteins in a subgroup shared at least one amino acid motif outside of the MYB domain. The red dots after the ginkgo sequence names indicate the gene expression profile by qRT-PCR performed in this study (color figure online)

transcript level of these genes by qRT-PCR after treatment with high salinity, cold (4 °C), heat (40 °C), abscisic acid (ABA), salicylic acid (SA), methyl jasmonate (MeJa), and ethephon (ET). As shown in Fig. 4, among the 8 candidate genes, *GbMYB15* and -26 were induced by all 7 treatments at varying levels, *GbMYB31* was induced by 5 treatments, and the other 5 genes were induced by 6 treatments. All candidate *GbMYBs* were induced by heat stress, and 5

GbMYBs (*GbMYB14*, -15, -26, -30, and -42) were induced by cold stress. *GbMYB14*, -30, and -42 were up-regulated by heat treatment but down-regulated by cold. In contrast, following both cold and heat stress, the expression level of *GbMYB15* was up-regulated, but *GbMYB26* showed the opposite results. Five *GbMYBs* (*GbMYB5*, -8, -15, -26, and -31) were significantly up-regulated in response to NaCl, and the expression of *GbMYB14*, -30 and -42 remained

Fig. 3 Relative expression levels of *GbMYB* genes in different tissues. The Y-axis indicates the normalized fold change in expression of each *GbMYB* gene. Error bars represented the standard error of the mean

Fig. 4 Expression of 8 *GbMYB* genes under various abiotic stresses. The expression heat map shows the qRT-PCR analysis results of *GbMYB* genes with cold (4 °C), heat (40 °C), NaCl, ABA, SA, ET and MeJa treatments for 0, 3, 6, 12, 24 and 48 h. The number of color scale indicates the normalized fold change in expression of each *GbMYB* gene (color figure online)

constant under NaCl stress. Except for *GbMYB31*, which was not induced by MeJa treatment, the other candidate MYB genes were significantly affected by the phytohormones ABA, SA, MeJa and ET. Interestingly, all *GbMYBs* were up-regulated following SA and ET treatment, and the expression of these *GbMYBs*, except *GbMYB 15*, was also up-regulated by ABA.

Discussion

Characterization of R2R3-MYB TFs in *G. biloba*

R2R3-MYB proteins are the largest group of MYB TFs and appear to be specific to plants and yeast (Jin and Martin 1999). Many R2R3-MYB gene families have been identified

in various sequenced plant species, and there are a different number of members in various species (Dubos et al. 2010; He et al. 2016; Li and Lu 2014; Stracke et al. 2001, 2014; Wang et al. 2015). Genome-wide bioinformatics analyses and functional studies have provided abundant data on *R2R3-MYB* families in plants. Although many studies have examined plant *R2R3-MYB* families, few members of this family have been characterized in gymnosperms. Additionally, little data are available on the size of the *R2R3-MYB* family and the structural features of these proteins in gymnosperms. Thus, it is difficult to characterize this family in the non-sequenced organism *G. biloba*. In this study, we used *Arabidopsis* as a reference for identifying *GbMYBs* from the transcriptome of *G. biloba*. Forty-five *GbMYB* genes with classic MYB domains were identified from the full-transcriptome data of ginkgo. The analysis indicated a small MYB family, which may due to genomic duplications or the limitation of data sources used in the study. More precise protein analyses need to be performed with the results from whole genome sequencing studies in the future.

Our results showed that the DBD domains of ginkgo MYBs are highly conserved, while the C-terminal sequences vary considerably, which is consistent with the results from other plant MYBs (Dubos et al. 2010; Stracke et al. 2001). Among the DBD domains, the distribution of conserved amino acids in ginkgo and *Arabidopsis* was quite similar with a few exceptions, indicating that this domain is highly conserved across plant MYB genes (Fig. 1). The major structure of the DBD for ginkgo is -W-x(19)-W-x(19)-W-x(12)-F/I-x(18)-W-x(18)-W-. In position 59 of the *GbMYBs*, Trp was substituted with the nonpolar amino acids Phe (61%), Ile (33%), Leu (4%), and Val (2%). These substitute residues have a similar hydrophobicity to Trp and may have few effects on the hydrophobic core of the MYB domain. The other conserved residues in the MYB domain were found to be predominantly concentrated in the third helix, suggesting that helix3 is more conserved than helix1 and helix2. The highly conserved helix3 sequence in ginkgo may indicate its functional conservation, and the residue substitutions in helix3 may affect the DNA recognition.

Phylogenetic relationship and functions in various biological processes

Phylogenetic-based classification of genes into subfamilies is supported by the gene structure and domain analysis. In general, members of the same subfamily often share similar sequences, conserved motifs and even binding partners, and they are likely to share similar functions (Singh et al. 2014; Wu et al. 2003a). Therefore, when lacking sufficient data, functional characterization of unknown proteins is

generally carried out based on comparisons with annotated proteins (Cao et al. 2013; Stracke et al. 2014; Tombuloglu et al. 2013). The *R2R3-MYBs* in *Arabidopsis* have been well studied and were used to predict the possible functions of the identified ginkgo MYBs. We performed a phylogenetic analysis of *GbMYBs* and *AtMYBs* and divided the 168 proteins into 26 subgroups. For many MYB proteins, no motif was found in C-terminal to group them. Twenty-nine of the 43 *GbMYBs* were divided into different subgroups, and 27 *GbMYBs* were classified in the same subgroup as the *Arabidopsis* proteins, indicating the evolutionary conservation of plant MYB proteins. One new *R2R3-MYB* subgroup, S26, in ginkgo was identified and indicated that these two proteins may have species-specialized functions that were either lost in *Arabidopsis* or were acquired after divergence through evolution (Dubos et al. 2010; Matus et al. 2008).

Members of the plant MYB gene family have a unique expression pattern in different tissues or under various treatments, indicating diverse or similar functions in different cases. Several MYB genes were expressed in specific tissues and are believed to be involved in plant growth and development, while others were expressed in a tissue-specific manner after treatment or stimuli (Chen et al. 2006; Kranz et al. 1998; Stracke et al. 2007). For example, the *Arabidopsis AtMYB21* gene was specifically expressed in flower buds, and *AtMYB26* and *AtMYB103* were primarily expressed in anthers (Higginson et al. 2003; Shin et al. 2002; Steiner-Lange et al. 2003). *AtMYB19* transcripts were only detected after infection with *Pseudomonas syringae* (Kranz et al. 1998). In this study, of the eight candidate *GbMYB* genes, four *GbMYBs* showed higher expression in the reproductive organs (*GbMYB8*, -14, -15, and -42), suggesting that these genes may be involved in the development of flowering.

GbMYB15 and *GbMYB16* were found to be MIXTA-like TFs that contain a conserved motif of AQWE-SARxxAExRLxRES (motif 11) and were classified in subgroup 9 with *AtMYB16*, -17, -106 (Bedon et al. 2014). MIXTA-like TFs are known to regulate trichome development and epidermal cell differentiation and have a putative role in early inflorescence development (Baumann et al. 2007; Jakoby et al. 2008; Perez-Rodriguez et al. 2005; Zhang et al. 2009). S13 contains *GbMYB4*, -11, -14, and -38 and *AtMYB50*, -55, -61, and -86, and the *AtMYBs* in this subgroup are involved in the regulation of lignin deposition, stomatal aperture, and responses to abiotic stresses (Liang et al. 2005; Newman et al. 2004; Shin et al. 2002). *GbMYB14* had the highest expression in gynoecia, suggesting a possible role in the lignin deposition of female flowers. *GbMYB12*, -19, -22, and -42 and their counterparts *AtMYB52*, -54, -69, -105, and -117 were grouped in S21, which predominantly includes positive regulators for

secondary wall thickening that function in lateral organ separation and axillary meristem formation (Lee et al. 2009; Zhong et al. 2008). *GbMYB42* had the highest expression in stamens, indicating that it may be involved in the differentiation of staminate flowers. *GbMYB7*, -8, -9, and -20 and *AtMYB44*, -70, -73, and -77 were grouped in S22, which has *AtMYBs* associated with stress responses (Jung et al. 2008). S18 contains *GbMYB35*, -37, and 7 *AtMYBs* are grouped into S18. The *Arabidopsis* members are predominantly involved in flowering and anther development (Allen et al. 2007; Gocal et al. 2001; Li and Lu 2014). In addition, *AtMYB41* and *AtMYB102* in S11 have been suggested to have a role in cell expansion and integration of signals derived from wounding and osmotic stresses, respectively (Cominelli et al. 2008; Denekamp and Smeekens 2003).

Candidate *GbR2R3-MYB* genes involved in flavonoid biosynthesis

Flavonoids are important secondary metabolites that serve a variety of developmental and defensive functions in plants (Hichri et al. 2011). They accumulate in all tissues of plants at different developmental stages. Flavonoid biosynthesis is predominantly regulated by TFs at the transcriptional level of the structural genes, depending on the environmental conditions (Xu et al. 2014). In *Arabidopsis*, *R2R3-MYBs* that control flavonoid biosynthesis have been characterized. They appear to be distributed in specific subgroups, including S3, S4, S6, S7 and S17, with different expression patterns (Dubos et al. 2010; Zhou et al. 2009). Seven *GbMYBs* are clustered into the same subgroup with the *AtMYBs* that are involved in flavonoid biosynthesis, including *GbMYB1*, -3, -21, -26, -31, -33, and -39. This indicates that *G. biloba* genes belonging the same subgroup are potential regulators of flavonoid biosynthesis. We also found that *GbMYB26* and *GbMYB31* were predominantly expressed in the leaf and gynoecia samples, suggesting that their possible regulatory role of flavonoid biosynthesis likely occurs in the leaves and gynoecia.

Moreover, *GbMYB1*, -3, -21, -31, and -39 contain the bHLH-binding consensus motif, which functions in the interaction between MYB and bHLH, suggesting that specific bHLH cofactors are required to act as regulatory factors, similar to other plant MYBs. The MYB proteins in plants containing the bHLH motif usually regulate flavonoid biosynthesis, and specific R/B-like bHLH proteins are needed to act as regulatory factors (Espley et al. 2007; Zhao et al. 2013; Zimmermann et al. 2004). The interaction between *R2R3-MYB* and bHLH controls the enzyme activity of flavonoid biosynthesis and thus alters the level of flavonoid production (Koes et al. 2005; Nakatsuka et al. 2009). A study from Xu et al. (2014) showed that *GbMYB0*

is closely related to the repressor *R2R3-MYB* subfamily involved in flavonoid biosynthesis and may repress transcription of key regulators of flavonoid biosynthesis in *G. biloba*. *GbMYB5* and *GbMYB13* were grouped in the same cluster (S8) with *GbMYB0*, indicating that these two *GbMYBs* may also be involved in flavonoid biosynthesis processes. Additionally, the deduced amino acid sequence of *GbMYB5* is highly similar to that of *GbMYB0*, with a 99.1% homology. Therefore, the phylogenetic and bioinformatics analyses showed that 8 *GbMYB* genes, including *GbMYB1*, -3, -5, -21, -26, -31, -33, and -39, are possibly involved in the regulation of flavonoid biosynthesis. We should consider these *GbMYB* genes as candidate regulators of flavonoid biosynthesis for further functional analysis.

Candidate stress-responsive *R2R3-MYB* genes in *G. biloba*

Because plants are sessile organisms, they are directly exposed to common adverse environmental factors that are known to induce oxidative stress, and they must develop a broad range of complex defense systems to adapt (Bohnert et al. 1995; Mittova et al. 2004). Elucidating the stress perception, signaling, and response mechanisms of plants is essential to understanding how they improve their stress tolerance in environmentally demanding conditions. In recent years, accumulated evidence has indicated that numerous *R2R3-MYB* genes could enhance plant tolerance to biotic and abiotic stresses, such as salinity, cold, heat, drought and wounding. This is predominantly mediated by regulating the expression of stress-related genes and subsequently altering multiple signal transduction pathways and metabolic processes (Xie et al. 2014). Although the mechanisms of *R2R3-MYBs* in specific metabolic pathway and stress tolerance have been well studied in *Arabidopsis*, the functions of the homologous *R2R3-MYB* genes are still unclear in most plants, particularly in gymnosperm. Gene expression profile analysis is useful for predicting the biological functions of putative proteins in ginkgo.

In this study, salinity, heat and cold were chosen for stress treatments. Salinity is one of the most severe abiotic stresses limiting plant productivity, and high salt conditions can affect various physiological processes from seed germination to plant development (Pasternak 1987). Temperature is another important factor that can restrict plant distribution, growth and development. High or low temperatures that exceed the plant's tolerance range are characterized as an abiotic stress factor. In *Arabidopsis*, 44.67 and 56.85% of 197 *MYB* genes were down-regulated, and 47.21 and 35.02% of *MYB* genes were up-regulated in cold and salt stress, respectively (Katiyar et al. 2012). In ginkgo, all candidate *GbMYBs* were regulated by at least two

stressors, indicating that *R2R3-MYBs* in ginkgo are also a stress-responsive family. The expression of different *GbMYBs* showed different patterns under salt and extreme temperature stresses. In general, overexpressing *R2R3-MYBs* produces tolerance to abiotic stresses; however, several of these genes serve as negative regulatory factors and have the opposite function. For example, *AtMYB15* is up-regulated by cold stress, but its overexpression negatively regulates cold tolerance, and it also leads to an increase in drought and salt tolerance (Agarwal et al. 2006; Ding et al. 2009). Although the abiotic stress-related *AtMYBs* have been well characterized, the mechanisms of how they respond to stress signals and regulate the expression of downstream genes have rarely been studied.

Signaling molecules, including ABA, SA, MeJa and ET, in higher plants play key roles in activating plant defense mechanisms in response to biotic and abiotic stresses, as well as participating in biological development (Cutler et al. 2010; Dong 1998; Fujita et al. 2006; Wasternack and Hause 2002). The signal pathway of ABA predominantly responds to abiotic stresses, such as drought, cold, and osmotic stress and also regulates various growth and developmental processes. In contrast, SA, MeJa and ET play essential roles in biotic stress signaling against pathogen infection (Fujita et al. 2006). Plant *R2R3-MYB* proteins involved in hormonal responses have been reported during growth and development processes and are often related to abiotic and biotic stress tolerance (Ambawat et al. 2013). Our study showed that almost all of the 8 *GbMYBs* were induced by the four phytohormones (ABA, SA, MeJa and ET), indicating that these *GbMYBs* are strongly induced by a wide range of signal molecules and may be involved in crosstalk between signal transduction pathways in response to various stresses. Several *AtMYBs* were found to be involved in crosstalk between different stresses. For example, *AtMYB96* is a key regulator in biotic and abiotic stress by linking ABA-mediated signals with SA biosynthesis and pathogen resistance response (Seo and Park 2010). *AtMYB44* regulated ABA-mediated stomatal closure in response to abiotic stresses, and its overexpression enhanced tolerance to salt and drought stresses, as well as *Pseudomonas syringae* resistance (Jung et al. 2008; Liu et al. 2010). Thus, in addition to putative roles in biological processes, the *GbMYBs* may also play complicated and crucial roles in response to biotic and abiotic stress. Further studies are required to elucidate the molecular mechanism underlying the involvement of the *GbMYBs* in ginkgo tolerance to biotic and abiotic stress.

In conclusion, we identified 42 full-length *R2R3-MYB* genes for the first time from the RNA-seq data of ginkgo. Although the distribution of amino acid residues at various positions was different between *GbMYBs* and *AtMYBs*, their conserved residues were quite similar. The conserved

domains of the *GbR2R3-MYB* genes were analyzed and provided additional evidence for the subgroup definition. Based on the phylogenetic analysis, the *GbMYBs* and *AtMYBs* were divided into distinct functional categories, and their putative roles were predicted. *GbMYBs* in S4, S7, S8 and S17 are potential regulators of flavonoid biosynthesis. The qRT-PCR analysis showed that *GbMYBs* also act as stress-responsive genes in ginkgo. These phylogenetic and expression analyses will be useful for elucidating the gene functions, and the characterization and validation of their roles are needed in the future.

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References

- Agarwal M, Hao Y, Kapoor A, Dong C, Fujii H, Zheng X, Zhu J (2006) A *R2R3* type MYB transcription factor is involved in the cold regulation of *CBF* genes and in acquired freezing tolerance. *J Biol Chem* 281:37636–37645
- Allen RS, Li J, Stahle MI, Dubroué A, Gubler F, Millar AA (2007) Genetic analysis reveals functional redundancy and the major target genes of the *Arabidopsis* miR159 family. *Proc Natl Acad Sci* 104:16371–16376
- Ambawat S, Sharma P, Yadav NR, Yadav RC (2013) MYB transcription factor genes as regulators for plant responses: an overview. *Ann N Y Acad Sci* 19:307–321
- Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS (2009) MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res* 37(Web Server issue):W202–W208
- Baumann K, Perez-Rodriguez M, Bradley D, Venail J, Bailey P, Jin H, Koes R, Roberts K, Martin C (2007) Control of cell and petal morphogenesis by *R2R3* MYB transcription factors. *Development* 134:1691–1701
- Bedon F, Schiefelbein J, Pang M (2014) Members of the *MYBMIXTA-like* transcription factors may orchestrate the initiation of fiber development in cotton seeds. *Front Plant Sci* 5:72–75
- Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. *Plant Cell* 7:1099–1111
- Cannon SB, Mitra A, Baumgarten A, Young ND, May G (2004) The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biol* 4:10
- Cao Z, Zhang S, Wang R, Zhang R, Hao Y (2013) Genome wide analysis of the apple MYB transcription factor family allows the identification of *MdoMYB121* gene conferring abiotic stress tolerance in plants. *PLoS ONE* 8:e69955
- Chen Y, Yang X, He K, Liu M, Li J, Gao Z, Lin Z, Zhang Y, Wang X, Qiu X, Shen Y, Zhang L, Deng X, Luo J, Deng X, Chen Z, Gu H, Qu L (2006) The MYB transcription factor superfamily of *Arabidopsis*: expression analysis and phylogenetic comparison with the rice MYB family. *Plant Mol Biol* 60:107–124
- Cheng H, Li L, Cheng S, Cao F, Wang Y, Yuan H (2011) Molecular cloning and function assay of a chalcone isomerase gene (*GbCHI*) from *Ginkgo biloba*. *Plant Cell Rep* 30:49–62

- Cheng H, Li L, Xu F, Cheng S, Cao F, Wang Y, Yuan H, Jiang D, Wu C (2013) Expression patterns of a cinnamyl alcohol dehydrogenase gene involved in lignin biosynthesis and environmental stress in *Ginkgo biloba*. *Plant Mol Biol* 40:707–721
- Cominelli E, Sala T, Calvi D, Gusmaroli G, Tonelli C (2008) Overexpression of the *Arabidopsis* *AtMYB41* gene alters cell expansion and leaf surface permeability. *Plant J* 53:53–64
- Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. *Annu Rev Plant Biol* 61:651–679
- DeFeudis FV, Drieu K (2000) *Ginkgo biloba* extract (EGb 761) and CNS functions basic studies and clinical applications. *Curr Drug Targets* 1:25–58
- DeFeudis FV, Papadopoulos V, Drieu K (2003) *Ginkgo biloba* extracts and cancer: a research area in its infancy. *Fundam Clin Pharmacol* 17:405–417
- Denekamp M, Smeekens SC (2003) Integration of wounding and osmotic stress signals determines the expression of the *AtMYB102* transcription factor gene. *Plant Physiol* 132:1415–1423
- Ding Z, Li S, An X, Liu X, Qin H, Wang D (2009) Transgenic expression of *MYB15* confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*. *J Genet Genomics* 36:17–29
- Dong X (1998) SA, JA, ethylene, and disease resistance in plants. *Curr Opin Plant Biol* 1:316–323
- Du H, Feng B, Yang S, Huang Y, Tang Y (2012) The R2R3-MYB transcription factor gene family in maize. *PLoS ONE* 7:1799–1823
- Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L (2010) MYB transcription factors in *Arabidopsis*. *Trends Plant Sci* 15:573–581
- Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *Plant J* 49:414–427
- Flagel LE, Wendel JF (2009) Gene duplication and evolutionary novelty in plants. *New Phytol* 183:557–564
- Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr Opin Plant Biol* 9:436–442
- Gocal GF, Sheldon CC, Gubler F, Moritz T, Bagnall DJ, MacMillan CP, Li SF, Parish RW, Dennis ES, Weigel D (2001) *GAMYB-like* genes, flowering, and gibberellin signaling in *Arabidopsis*. *Plant Physiol* 127:1682–1693
- Gong Y, Liao Z, Guo B, Sun X, Tang K (2006) Molecular cloning and expression profile analysis of *Ginkgo biloba* *DXS* gene encoding 1-deoxy-D-xylulose 5-phosphate synthase, the first committed enzyme of the 2-C-methyl-D-erythritol 4-phosphate pathway. *Planta Med* 72:329–335
- Goral TK, Johnson MP, Duffy CD, Brain AP, Ruban AV, Mullineaux CW (2012) Light-harvesting antenna composition controls the macrostructure and dynamics of thylakoid membranes in *Arabidopsis*. *Plant J* 69:289–301
- He Q, Jones DC, Li W, Xie F, Ma J, Sun R, Wang Q, Zhu S, Zhang B (2016) Genome-wide identification of R2R3-MYB genes and expression analyses during abiotic stress in *Gossypium raimondii*. *Sci Rep* 6:22980
- Hichri I, Barrieu F, Bogs J, Kappel C, Delrot S, Lauvergeat V (2011) Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. *J Exp Bot* 62:erq442
- Higginson T, Li SF, Parish RW (2003) *AtMYB103* regulates tapetum and trichome development in *Arabidopsis thaliana*. *Plant J* 35:177–192
- Hricova A, Fejer J, Libiakova G, Szabova M, Gazo J, Gajdosova A (2016) Characterization of phenotypic and nutritional properties of valuable *Amaranthus cruentus* L. mutants. *Turk J Agric For* 40:761–771
- Jakoby MJ, Falkenhahn D, Mader MT, Brininstool G, Wischnitzki E, Platz N, Hudson A, Hülskamp M, Larkin J, Schnittger A (2008) Transcriptional profiling of mature *Arabidopsis* trichomes reveals that *NOECK* encodes the *MIXTA-like* transcriptional regulator MYB106. *Plant Physiol* 148:1583–1602
- Jin H, Martin C (1999) Multifunctionality and diversity within the plant MYB-gene family. *Plant Mol Biol* 41:577–585
- Jung C, Seo JS, Han SW, Koo YJ, Kim CH, Song SI, Nahm BH, Do Choi Y, Cheong JJ (2008) Overexpression of *AtMYB44* enhances stomatal closure to confer abiotic stress tolerance in transgenic *Arabidopsis*. *Plant Physiol* 146:623–635
- Katiyar A, Smita S, Lenka SK, Rajwanshi R, Chinnusamy V, Bansal KC (2012) Genome-wide classification and expression analysis of MYB transcription factor families in rice and *Arabidopsis*. *BMC Genom* 13:544–582
- Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci* 10:236–242
- Kranz HD, Denekamp M, Greco R, Jin H, Leyva A, Meissner RC, Petroni K, Urzainqui A, Bevan M, Martin C (1998) Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. *Plant J* 16:263–276
- Lee D-K, Geisler M, Springer PS (2009) *LATERAL ORGAN FUSION1* and *LATERAL ORGAN FUSION2* function in lateral organ separation and axillary meristem formation in *Arabidopsis*. *Development* 136:2423–2432
- Li C, Lu S (2014) Genome-wide characterization and comparative analysis of R2R3-MYB transcription factors shows the complexity of MYB-associated regulatory networks in *Salvia miltiorrhiza*. *BMC Genom* 15:277
- Li L, Cheng H, Yuan H, Xu F, Cheng S, Cao F (2014) Functional characterization of the *Ginkgo biloba* chalcone synthase gene promoter in transgenic tobacco. *Genet Mol Res* 13:3446–3460
- Liang Y, Dubos C, Dodd IC, Holroyd GH, Hetherington AM, Campbell MM (2005) *AtMYB61*, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. *Curr Biol* 15:1201–1206
- Liao YL, Shen YB, Jie C, Zhang WW, Cheng SY, Feng X (2015) Isolation, expression, and promoter analysis of *GbWRKY2*: a novel transcription factor gene from *Ginkgo biloba*. *Int J Genomics* 2015:607185
- Lipsick JS (1996) One billion years of Myb. *Oncogene* 13:223–235
- Liu R, Lü B, Wang X, Zhang C, Zhang S, Qian J, Chen L, Shi H, Dong H (2010) Thirty-seven transcription factor genes differentially respond to a harpin protein and affect resistance to the green peach aphid in *Arabidopsis*. *J Biosci* 35:435–450
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408
- Matus JT, Aquea F, Arce-Johnson P (2008) Analysis of the grape MYB R2R3 subfamily reveals expanded wine quality-related clades and conserved gene structure organization across *Vitis* and *Arabidopsis* genomes. *BMC Plant Biol* 8:83
- Mittova V, Guy M, Tal M, Volokita M (2004) Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. *J Exp Bot* 55:1105–1113
- Nakatsuka A, Yamagishi M, Nakano M, Tasaki K, Kobayashi N (2009) Light-induced expression of basic helix-loop-helix genes involved in anthocyanin biosynthesis in flowers and leaves of Asiatic hybrid lily. *Sci Hortic* 121:84–91

- Newman LJ, Perazza DE, Juda L, Campbell MM (2004) Involvement of the R2R3-MYB, *AtMYB61*, in the ectopic lignification and dark-photomorphogenic components of the *det3* mutant phenotype. *Plant J* 37:239–250
- Novo-Uzal E, Gutiérrez J, Martínez-Cortés T, Pomar F (2014) Molecular cloning of two novel peroxidases and their response to salt stress and salicylic acid in the living fossil *Ginkgo biloba*. *Ann Bot* 114:923–936
- Ogata K, Hojo H, Aimoto S, Nakai T, Nakamura H, Sarai A, Ishii S, Nishimura Y (1992) Solution structure of a DNA-binding unit of Myb: a helix-turn-helix-related motif with conserved tryptophans forming a hydrophobic core. *Proc Natl Acad Sci* 89:6428–6432
- Ogata K, Morikawa S, Nakamura H, Sekikawa A, Inoue T, Kanai H, Sarai A, Ishii S, Nishimura Y (1994) Solution structure of a specific DNA complex of the Myb DNA-binding domain with cooperative recognition helices. *Cell* 79:639–648
- Ogata K, Morikawa S, Nakamura H, Hojo H, Yoshimura S, Zhang R, Aimoto S, Ametani Y, Hirata Z, Sarai A (1995) Comparison of the free and DNA-complexed forms of the DNA-binding domain from c-Myb. *Nat Struct Mol Biol* 2:309–320
- Ogata K, Kanei-Ishii C, Sasakiz M, Hatanaka H, Nagadoiz A, Enariz M, Nakamura H, Nishimura Y (1996) The cavity in the hydrophobic core of Myb DNA-binding domain is reserved for DNA. *Nat Struct Biol* 3:178–187
- Pasternak D (1987) Salt tolerance and crop production—a comprehensive approach. *Annu Rev Phytopathol* 25:271–291
- Paz-Ares J, Ghosal D, Wienand U, Peterson P, Saedler H (1987) The regulatory *c1* locus of *Zea mays* encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. *EMBO J* 6:3553–3558
- Perez-Rodriguez M, Jaffe FW, Butelli E, Glover BJ, Martin C (2005) Development of three different cell types is associated with the activity of a specific MYB transcription factor in the ventral petal of *Antirrhinum majus* flowers. *Development* 132:359–370
- Rabinowicz PD, Braun EL, Wolfe AD, Bowen B, Grotewold E (1999) Maize R2R3 Myb genes: sequence analysis reveals amplification in the higher plants. *Genetics* 153:427–444
- Riechmann JL, Heard J, Martin G, Reuber L, Jiang CZ, Keddie J, Adam L, Pineda O, Ratcliffe O, Samaha R (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science* 290:2105–2110
- Rosinski JA, Atchley WR (1998) Molecular evolution of the Myb family of transcription factors: evidence for polyphyletic origin. *J Mol Evol* 46:74–83
- Royer DL, Hickey LJ, Wing SL (2003) Ecological conservatism in the “living fossil” *Ginkgo*. *J Inf* 29:84–104
- Sakar E, Unver H, Bakir M, Ulas M, Sakar ZM (2016) Genetic relationships among olive (*Olea europaea* L.) cultivars native to Turkey. *Biochem Genet* 54:348–359
- Saridas MA, Kafkas NE, Zarifikhosroshahi M, Bozhaydar O, Kargi SP (2016) Quality traits of green plums (*Prunus cerasifera* Ehrh.) at different maturity stages. *Turk J Agric For* 40:655–663
- Seo PJ, Park CM (2010) MYB96-mediated abscisic acid signals induce pathogen resistance response by promoting salicylic acid biosynthesis in *Arabidopsis*. *New Phytol* 186:471–483
- Shen G, Pang Y, Wu W, Liao Z, Zhao L, Sun X, Tang K (2006) Cloning and characterization of a root-specific expressing gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Ginkgo biloba*. *Mol Biol Rep* 33:117–127
- Shin B, Choi G, Yi H, Yang S, Cho I, Kim J, Lee S, Paek NC, Kim JH, Song PS (2002) *AtMYB21*, a gene encoding a flower-specific transcription factor, is regulated by COP1. *Plant J* 30:23–32
- Singh UM, Chandra M, Shankhdhar SC, Kumar A (2014) Transcriptome wide identification and validation of calcium sensor gene family in the developing spikes of finger millet genotypes for elucidating its role in grain calcium accumulation. *PLoS ONE* 9:e103963
- Solmaz I, Kacar Y, Simsek O, Sari N (2016) Genetic characterization of turkish snake melon (*Cucumis melo* L. subsp. *melo flexuosus* Group) accessions revealed by SSR markers. *Biochem Genet* 54:534–543
- Steiner-Lange S, Unte US, Eckstein L, Yang C, Wilson ZA, Schmelzer E, Dekker K, Saedler H (2003) Disruption of *Arabidopsis thaliana* MYB26 results in male sterility due to non-dehiscent anthers. *Plant J* 34:519–528
- Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. *Curr Opin Plant Biol* 4:447–456
- Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K, Weisshaar B (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. *Plant J* 50:660–677
- Stracke R, Holtgräwe D, Schneider J, Pucker B, Sörensen TR, Weisshaar B (2014) Genome-wide identification and characterisation of R2R3-MYB genes in sugar beet (*Beta vulgaris*). *BMC Plant Biol* 14:249
- Tombuloglu H, Kekec G, Sakcali MS, Unver T (2013) Transcriptome-wide identification of R2R3-MYB transcription factors in barley with their boron responsive expression analysis. *Mol Genet Genomics* 288:141–155
- Wang Z, Tang J, Hu R, Wu P, Hou X, Xiong A (2015) Genome-wide analysis of the R2R3-MYB transcription factor genes in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) reveals their stress and hormone responsive patterns. *BMC Genom* 16:17
- Wasternack C, Hause B (2002) Jasmonates and octadecanoids: signals in plant stress responses and development. *Prog Nucleic Acid Res Mol Biol* 72:165–221
- Wilkins O, Nahal H, Foong J, Provart NJ, Campbell MM (2009) Expansion and diversification of the *Populus* R2R3-MYB family of transcription factors. *Plant Physiol* 149:981–993
- Wu C, Huang H, Yeh L-SL, Barker WC (2003a) Protein family classification and functional annotation. *Comput Biol Chem* 27:37–47
- Wu X, Lim SH, Yang W (2003b) Characterization, expression and phylogenetic study of R2R3-MYB genes in orchid. *Plant Mol Biol* 51:959–972
- Xie R, Zheng L, Deng L, He S, Yi S, Lv Q, Zheng Y (2014) The role of R2R3MYB transcription factors in plant stress tolerance. *J Anim Plant Sci* 24:1821–1833
- Xu F, Cheng H, Cai R, Li LL, Chang J, Zhu J, Zhang FX, Chen LJ, Wang Y, Cheng SH (2008) Molecular cloning and function analysis of an anthocyanidin synthase gene from *Ginkgo biloba*, and its expression in abiotic stress responses. *Mol Cells* 26:536–547
- Xu F, Li L, Zhang W, Cheng H, Sun N, Cheng S, Wang Y (2012) Isolation, characterization, and function analysis of a flavonol synthase gene from *Ginkgo biloba*. *Mol Biol Rep* 39:2285–2296
- Xu F, Ning Y, Zhang W, Liao Y, Li L, Cheng H, Cheng S (2014) An R2R3-MYB transcription factor as a negative regulator of the flavonoid biosynthesis pathway in *Ginkgo biloba*. *Funct Integr Genomics* 14:177–189
- Zhang Y, Cao G, Qu L, Gu H (2009) Characterization of *Arabidopsis* MYB transcription factor gene *AtMYB17* and its possible regulation by LEAFY and AGL15. *J Genet Genomics* 36:99–107
- Zhang L, Chen J, Li Q, Chen W (2016) Transcriptome-wide analysis of basic helix-loop-helix transcription factors in *Isatis indigotica* and their methyl jasmonate responsive expression profiling. *Gene* 576:150–159
- Zhao L, Gao L, Wang H, Chen X, Wang Y, Yang H, Wei C, Wan X, Xia T (2013) The R2R3-MYB, bHLH, WD40, and related transcription factors in flavonoid biosynthesis. *Funct Integr Genomics* 13:75–98

- Zhong R, Lee C, Zhou J, McCarthy RL, Ye Z (2008) A battery of transcription factors involved in the regulation of secondary cell wall biosynthesis in *Arabidopsis*. *Plant Cell* 20:2763–2782
- Zhou J, Lee C, Zhong R, Ye Z (2009) MYB58 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in *Arabidopsis*. *Plant Cell* 21:248–266
- Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF (2004) Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. *Plant J* 40:22–34