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Genome-Wide Analysis and Expression Profiles of the *MYB* Genes in *Brachypodium distachyon*

Shoukun Chen, Xin Niu, Yuxiang Guan and Haifeng Li*

State Key Laboratory of Crop Stress Biology for Arid Areas, College of Agronomy, Northwest A&F University, Yangling, China
*Corresponding author: E-mail, lhf@nwsuaf.edu.cn
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MYB transcription factors are widespread in plants and play key roles in plant development. Although MYB transcription factors have been thoroughly characterized in many plants, genome-wide analysis of the *MYB* gene family has not yet been undertaken in *Brachypodium distachyon*. In this study, 122 BdMYB transcription factors were identified, comprising 85 MYB-R2R3, 34 MYB-related and three MYB-R1R2R3. Phylogenetic analysis showed that BdMYBs, OsMYBs and AtMYBs with similar functions were clustered in the same subgroup, and the phylogenetic relationships of BdMYB transcription factors were supported by highly conserved motifs and gene structures. Two *cis*-elements were found in the promoters of *BdMYB* genes. One is related to plant growth/development, the other is related to stress responses. Gene Ontology (GO) analysis indicated that most of the *BdMYB* genes are involved in various biological processes. The chromosome distribution pattern strongly indicated that genome-wide tandem and segment duplication mainly contributed to the expansion of the *BdMYB* gene family. Synteny analysis showed that 56, 58 and 61 *BdMYB* genes were orthologous to rice, maize and sorghum, respectively. We further demonstrated that *BdMYB* genes have evolved under strong purifying selection. The expression profiles indicated that most *BdMYB* genes might participate in floral development and respond to abiotic stresses. Additionally, 338 pairs of proteins were predicted to interact by constructing the interaction network. This work laid the foundation and provided clues for understanding the biological functions of these transcription factors.

Keywords: *Brachypodium distachyon* • Expression profiles • MYB • Transcription factor.

Abbreviations: ABRE, ABA-responsive element; CDS, coding domain sequence; EST, expressed sequence tag; GO, Gene Ontology; HMM, hidden Markov model; HSE, heat shock element; Ka, non-synonymous substitution rate; Ks, synonymous substitution rate; MeJA, methyl jasmonate; Mya, million years ago; NJ, Neighbor–Joining; qRT-PCR, quantitative real-time PCR; SA, salicylic acid; TF, transcription factor.

Introduction

When plants encounter diverse environmental stresses, they generate a series of physiological and biochemical responses, as well as a complex of signaling transduction pathways (Yamaguchi-Shinozaki and Shinozaki 2006). After suffering from stresses, many transcription factors (TFs) participate in the response to these environmental stresses as crucial regulators (Søren et al. 2013) by regulating the expression of stress-related genes (Zhu et al. 2015). Among these TFs, MYB (myeloblastosis) transcription factors are widely distributed in the plant kingdom and comprise one of the largest families (Katiyar et al. 2012).

The first *MYB* gene was the 'oncogene' v-*Myb* found in avian myeloblastosis virus (Klempnauer et al. 1982). Then, three v-*Myb*-related genes c-*Myb*, A-*Myb* and B-*Myb*, were identified in other eukaryotes and reported to function in the regulation of cell proliferation, differentiation and apoptosis (Lipsick 1996, Kranz et al. 1998, Weston 1998). The maize (*Zea mays*) *C1* gene was the first reported plant MYB TF and it was shown to be involved in regulation of anthocyanin pigmentation (Pazares et al. 1987). These MYB TFs are characterized by the presence of a MYB domain which is highly conserved at the N-terminus and consists of 1–4 repeats, named 1R-, 2R-, 3R- and 4R-MYB (Baldoni et al. 2015). Each repeat contains approximately 52 amino acid residues to form three α-helices. The second and third helices involve the formation of a helix–turn–helix (HTH) structure (Dubos et al. 2010). MYB-R1R2R3 proteins are common in animals, while the MYB-R2R3 proteins are more prevalent in plants (X. Li et al. 2016).

So far, a large number of *MYB* genes have been identified in different plants. For example, there are 155 in rice, 197 in Arabidopsis, 127 in tomato, 209 in foxtail millet, and so on (Katiyar et al. 2012, Muthamilarasan et al. 2014, Z. Li et al. 2016). MYB TFs were identified to function in many physiological and molecular processes, such as plant growth/development, secondary metabolism and signal transduction (Allan et al. 2008, Zhang et al. 2012). For example, *AtMYB94* and *AtMYB96* function redundantly in the biosynthesis of cuticular wax in Arabidopsis (Lee et al. 2016); another MYB-related Arabidopsis gene, *HHO2*, functions as a regulator in root development and controls phosphate homeostasis (Nagarajan et al. 2016); additionally, as co-activators, γMYB1 and γMYB2 bind to the *cis*-element of the *P1BS* gene and activate the expression of *PLA2-γ* (Nguyen et al. 2016). Recently, *LfMYB113*, a R2R3-MYB gene, was reported to be responsible for the coloration of autumn leaves in Formosan sweet gum (Wen and Chu 2017).

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In addition, transcriptome analyses showed that most BdMYB TFs responded to abiotic stresses, such as heat (Chen and Li 2016), drought (Verelst et al. 2013), high salinity (Priest et al. 2014) and hormone treatments (Kakei et al. 2015).

Brachypodium distachyon is a new model plant with characteristics such as a short life cycle, self-pollination and a small genome size of 272 Mb (International Brachypodium Initiative 2010). Up to now, several studies have been conducted to investigate *B. distachyon* TFs, including AP2/ERF, MADS-box, MAPK/MAPKK, NAC, TIFY and WRKY families (Wei et al. 2014, Wen et al. 2014, Zhang et al. 2015, Zhu et al. 2015, Cui et al. 2016, Feng et al. 2016), but a genome-wide analysis of the BdMYB TFs has not yet been undertaken. In this study, *BdMYB* genes were identified and a series of investigations such as on evolution, gene structure, conserved domains/motifs, duplication and the expression pattern were carried out. This research provided clues and laid the foundation for functional elucidation of these genes.

Results

Identification and classification of the *MYB* genes in *B. distachyon*

By using a hidden Markov model (HMM) search against the *B. distachyon* genome and the BLAST algorithm (see the Materials and Methods) with 155 and 197 MYB TFs in rice and Arabidopsis, respectively, a total of 122 putative BdMYB TFs were identified in *B. distachyon*, accounting for about 0.3894% of all annotated *B. distachyon* genes, which is similar to that of rice (0.3934%) and smaller than that of Arabidopsis (0.5964%) (Katiyar et al. 2012). The predicted *BdMYB* genes were named from *BdMYB001* to *BdMYB122* based on the co-ordinate order on *B. distachyon* chromosomes. To evaluate the existence of *BdMYB* genes which we identified, the CDSs (coding domain sequences) were extracted from *B. distachyon*, and were used to search against the *B. distachyon* expressed sequence tag (EST) database using the BLASTN tool. The results demonstrated that most *BdMYB* genes had one or more representative ESTs and only eight genes showed no EST hits. In order to understand the physical properties of BdMYB TFs, the characteristic features were also identified. All *BdMYB* genes varied greatly in length and physicochemical properties. The detailed information is listed in **Supplementary Table S1**.

To understand further the evolutionary relationship of MYB proteins in *B. distachyon*, rice and Arabidopsis, an unrooted Neighbor–Joining (NJ) phylogenetic tree was generated based on the full-length proteins (Katiyar et al. 2012). As shown in the phylogenetic tree (**Supplementary Fig. S1**), the MYB proteins were classified into three subgroups named MYB-related (XII–XVII), MYB-R2R3 (I–XI, XIX and XX) and MYB-R1R2R3 (XVIII) based on the presence of one, two or three MYB repeats in the DNA-binding domain, respectively. Additionally, an unrooted phylogenetic tree of BdMYB TFs was also constructed. As shown in **Fig. 1A**, there are 85 (69.67%) MYB-R2R3 TFs, 34 MYB-related TFs and three MYB-R1R2R3 TFs.

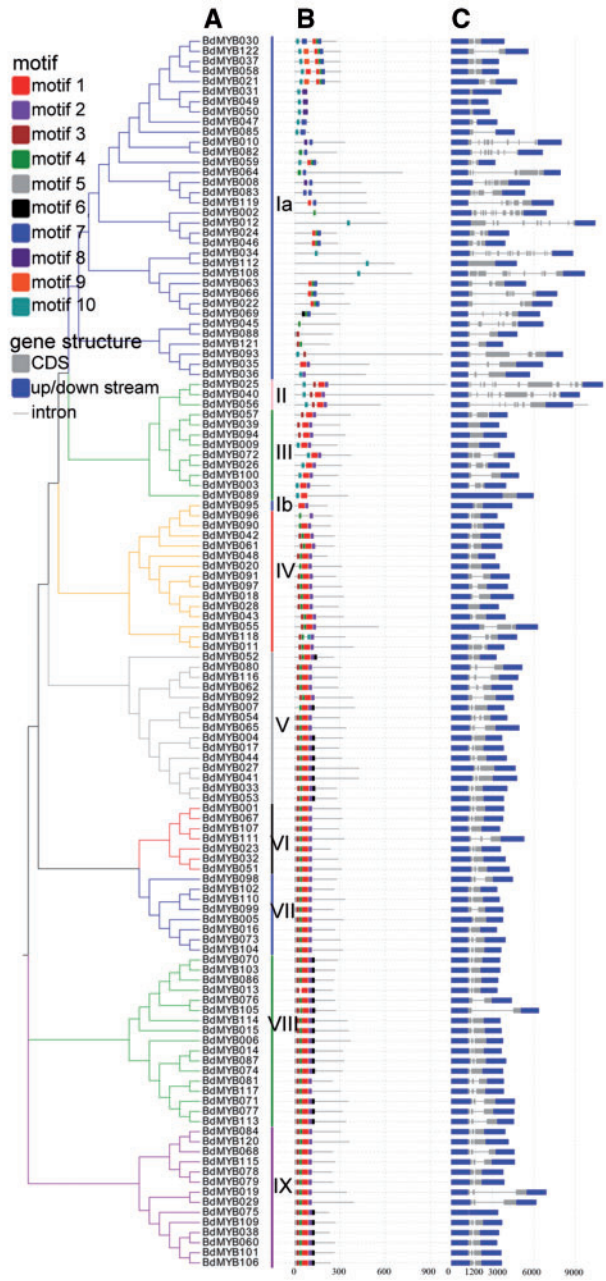

Fig. 1 Phylogenetic relationships (A), motif compositions (B) and gene structure (C) of BdMYBs. The tree was constructed with 1,000 bootstrap replications using MEGA7 based on the full-length protein sequence. The exon–intron structures of these genes were graphically displayed by the Gene Structure Display Server using the CDS and genome sequence of *BdMYB* genes. The protein sequences of BdMYB TFs were used to predict the conserved motifs by using the MEME Suite web server.

Conserved motifs of BdMYB TFs and structure of *BdMYB* genes

The MYB domain is the core of MYB TFs and binds to the promoter of their downstream genes. In total, 10 conserved motifs were identified and designated Motifs 1–10 (**Supplementary Fig. S2**). Among them, Motif 3 is the basic

region and the hinge of the MYB domain, while Motifs 6 and 8 only appear in MYB-R2R3 TFs; similarly, Motif 5 only appears in MYB-R2R3 TFs, except for its presence in the MYB-related TF BdMYB088. Motifs 7, 9 and 10 only appear in MYB-related TFs. In contrast, Motif 1 was found in most MYB-R1R2R3 and MYB-R2R3 TFs except BdMYB035. These results indicated the divergence of the BdMYB TFs (**Fig. 1B**).

Since the analyses of gene structure could help understand the gene functions, regulation and evolution (Feng et al. 2016), the structure of the *BdMYB* genes was also examined. The number of exons ranges from one to 16, with an average of 3.21. Most genes encoding MYB-related (group I, **Fig. 1A**) and MYB-R1R2R3 (group II, **Fig. 1A**) TFs contain more exons than genes encoding MYB-R2R3 TFs. Additionally, nine *BdMYB* genes (*BdMYB009*, *BdMYB028*, *BdMYB031*, *BdMYB039*, *BdMYB049*, *BdMYB050*, *BdMYB075*, *BdMYB094* and *BdMYB112*) only have one exon. Furthermore, the results showed that exon/intron structures of *BdMYB* genes in the same subfamilies were highly conserved, though the lengths of introns and exons were different (**Fig. 1C**).

Stress-related *cis*-elements in promoters of *BdMYB* genes and Gene Ontology (GO) annotation

To better predict the gene functions, we identified the *cis*-element within the 1.5 kb promoter region of *BdMYB* genes. As a result, two main types of *cis*-elements were detected: one type is related to plant growth/development and the other is related to stress responses (**Supplementary Table S2**). *Cis*-elements related to growth/development include light responsive (G-box, sp1 and ACE), endosperm expression (skn-1_motif), circadian control (circadian) and meristem-specific activation (O2-site). *Cis*-elements related to abiotic stresses include methyl jasmonate (MeJA) response (CGTCA-motif), ABA response [ABA-responsive element (ABRE)], salicylic acid (SA) response (TCA element), drought response (MBS), heat response [heat shock element (HSE)], low-temperature response (LTR), and so on.

Furthermore, GO assignments were used to predict the functions of BdMYB proteins by classifying them into various biological processes. TFs were divided into categories with three independent ontologies, i.e. biological process (BP), molecular function (MF) and cellular components (CC) (Ashburner et al. 2000). As shown in **Fig. 2**, the functions of BdMYB TFs related to biological process include biological regulation, cellular process, metabolic process, pigmentation and response to stimulus; the molecular function is to participate in binding to nucleic acids; the cellular component is to locate in the cell nucleus. These GO annotations of BdMYB proteins were in agreement with the experimental findings in Arabidopsis and rice (Katiyar et al. 2012, Smita et al. 2015).

Chromosomal location, gene duplication and synteny of *BdMYB* genes

To better understand the genomic distribution of *BdMYB* genes, their approximate positions on each chromosome were marked. As shown in **Fig. 3**, among the five chromosomes,

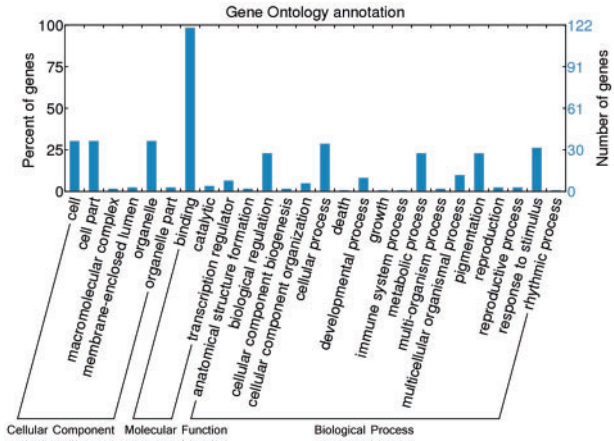

Fig. 2 Gene Ontology annotation of BdMYB proteins. BdMYB TFs were divided into categories with three independent ontologies, namely biological process, molecular function and cellular components.

Chr2 had 42 (34.43%) *BdMYB* genes, while Chr3, Chr4, Chr1 and Chr5 had 27, 22, 19 and 12 *BdMYB* genes, respectively. Unexpectedly, genes encoding MYB-R1R2R3 TFs were located in Chr2 without exception (**Supplementary Table S1**).

Furthermore, to investigate the gene duplication events in *B. distachyon*, tandem duplications and segmental duplications were also identified. Among the *BdMYB* genes, 26 tandem duplicated genes were identified and formed 16 pairs (**Supplementary Table S3**). These duplicated genes encode MYB-related or MYB-R2R3 TFs. Meanwhile, 34 gene pairs generated from chromosomal segmental duplications were found (**Fig. 3**; **Supplementary Table S4**). Combined with the phylogenetic tree (**Fig. 1A**), we found that the segment duplication gene pairs were clustered together into the MYB-R2R3 or MYB-related subgroups, with one exception (BdMYB035–BdMYB056). To explore further the evolutionary process of *BdMYB* genes, genome synteny among rice, maize and sorghum was also investigated (**Fig. 4A–C**) and the results showed that 56, 58 and 61 *BdMYB* genes had homologous genes in rice (**Supplementary Table S5**), maize (**Supplementary Table S6**) and sorghum (**Supplementary Table S7**), respectively.

In addition, the substitution rate (non-synonymous/synonymous, Ka/Ks) was an effective index to determine the positive selection pressure after duplication and was typically used to understand the direction of evolution and its selective strength in a coding sequence (Li et al. 2009). $Ka/Ks = 1$ stands for neutral selection, $Ka/Ks < 1$ means purifying selection, while $Ka/Ks > 1$ signifies positive selection (Lynch and Conery 2000). Therefore, we calculated the Ka, Ks and Ka/Ks of each gene pair. The average value of Ka or Ks for tandem duplication between *BdMYB* genes was significantly higher than the average for segmental duplication genes (**Fig. 4D, E**). Thus, it is not surprising to see that the Ka/Ks of tandem duplication genes (0.78) is much higher than for segmental duplication genes (0.46) (**Fig. 4F**). The results showed that the Ka/Ks values of gene pairs of *B. distachyon* and gene pairs of rice, maize and sorghum orthologs were < 1. The average Ka/Ks values between *B. distachyon* and maize, sorghum and rice

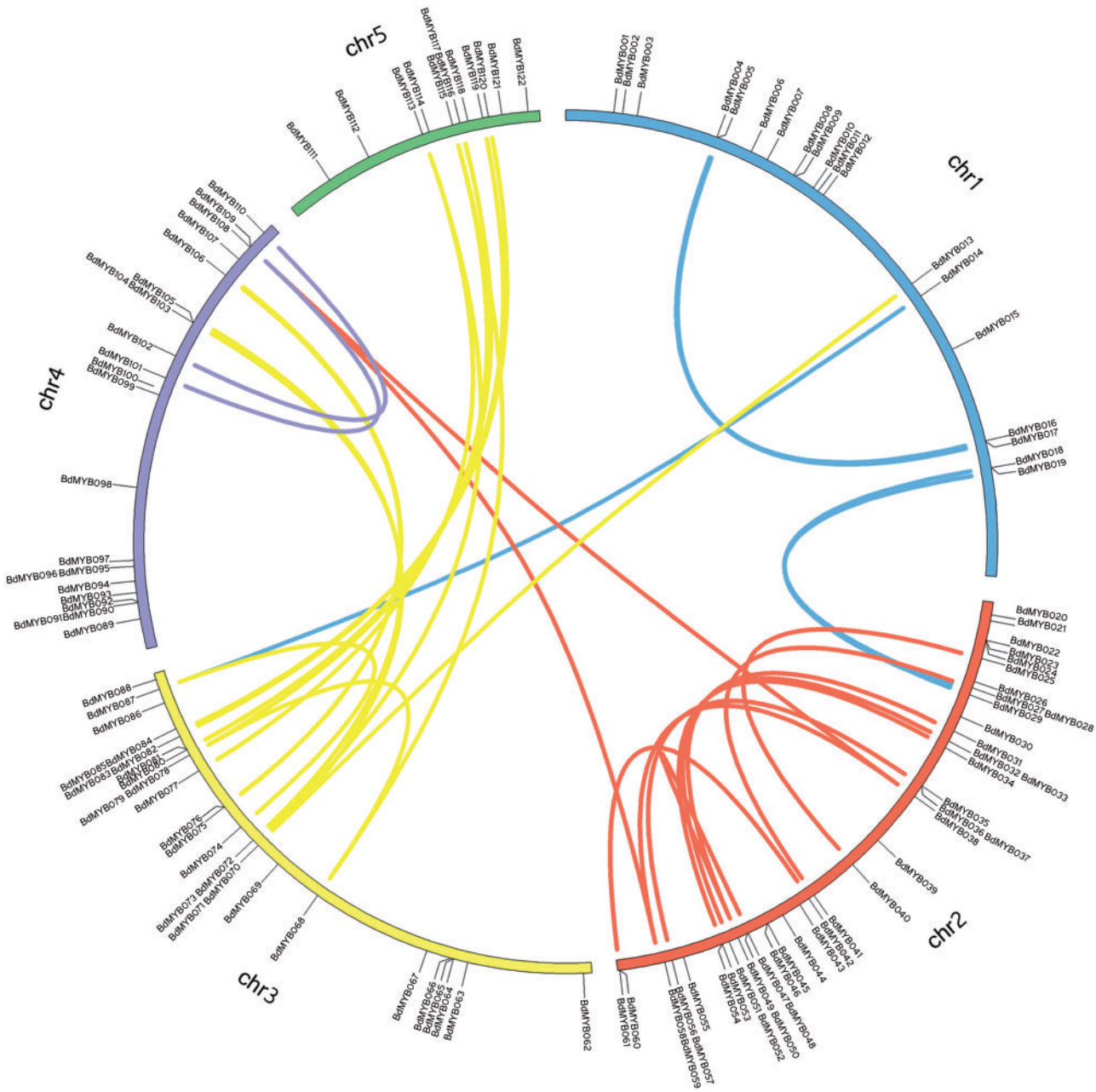

Fig. 3 Genomic locations of BdMYB TFs and segmentally duplicated gene pairs in the *B. distachyon* genome. A total of 122 BdMYB TFs were unevenly located in five chromosomes. There were 34 segmentally duplicated gene pairs identified in the *B. distachyon* genome.

were 0.4094, 0.3650 and 0.3581, respectively. Moreover, the duplication events of tandem and segmental duplication genes were evaluated to have occurred approximately 64 and 63 Mya (million years ago), respectively. The divergence time was about 46.5374, 52.9789 and 51.0968 Mya for rice, maize and sorghum, respectively. These results implied that the duplication events played a significant role in evolution and functional divergence of *BdMYB* genes, as well as other grass species.

The interaction network between BdMYB proteins and other proteins

To understand further the roles of BdMYB proteins, an interaction network of BdMYB proteins was built on the basis of Arabidopsis proteins to predict the relationship between BdMYB proteins and other proteins. Generally, 338 protein pairs were predicted with high confidence to interact (score $>$ 0.800) between 101 BdMYB proteins and 116 other *B. distachyon* proteins (**Supplementary Table S8**). Among these 338 protein pairs, 17.75% (60/338) have been verified by experiments in Arabidopsis.

Expression profiles of *BdMYB* genes

To obtain more insight into the temporal and spatial expression patterns of *BdMYB* genes, the expression profiles were analyzed to detect the tissue-specific expression of *BdMYB* genes by integrating the results of previous reports (Davidson et al. 2012).

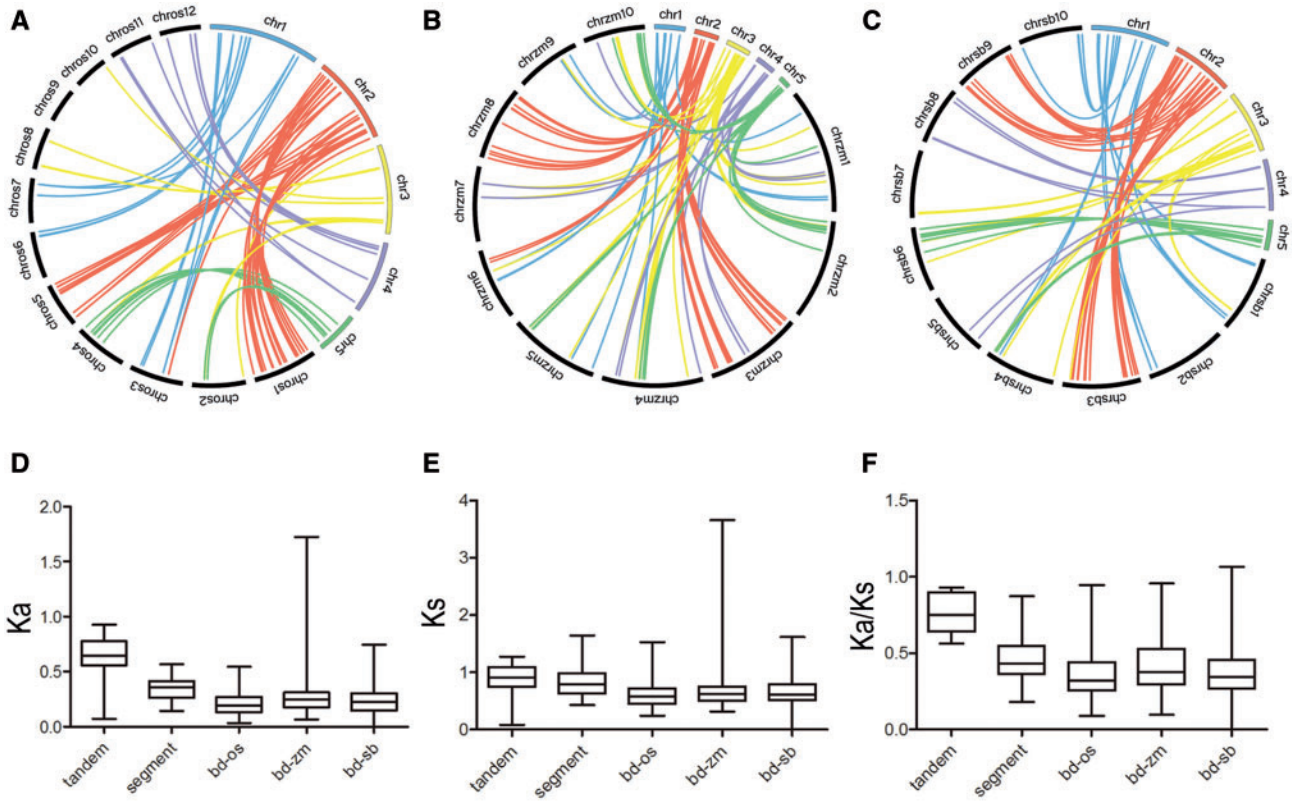

Fig. 4 Comparative physical mapping shows the orthologous relationships of BdMYB TFs with (A) rice, (B) maize and (C) sorghum. (D–F) Average values of Ka, Ks and Ka/Ks, respectively, of duplicated genes. There were 56, 58 and 61 *BdMYB* genes identified to be orthologous to rice (A), maize (B) and sorghum (C), respectively. The horizontal axes in (D–F) stand for tandem duplication (tandem), segmental duplication (segment) and the duplication between *B. distachyon* and rice (bd-os), maize (bd-zm) and sorghum (bd-sb), respectively.

A total of 35.25% *BdMYB* genes (43/122, including one *MYB*-R1R2R3, 17 *MYB*-R2R3 and 25 *MYB*-related genes) were highly expressed in all tissues, while some other genes displayed a tissue-specific expression pattern. For example, 19 *MYB*-R2R3 genes were found to be expressed mainly in flowers, while eight *BdMYB* genes were found to be expressed exclusively in leaves (**Fig. 5**) (Davidson et al. 2012).

To understand the roles of these TFs in response to abiotic stresses, expression profiles of *BdMYB* genes under different abiotic stresses were also examined. The results showed that 99.18% (121/122) were up- or down-regulated by hormone treatment, 47.54% (58/122) were up-regulated by ABA and 48.36% (59/122) were up-regulated by ethylene (**Fig. 6A**) (Kakei et al. 2015). In addition, the results also showed that 54.10% (66/122) of *BdMYB* genes were in response to single salinity, drought and heat stress, or their double and triple stress combinations (**Fig. 6B**). Among these 66 *BdMYB* genes, 12 were up-regulated while 19 were down-regulated (Shaar-Moshe and Blumwald 2017).

We also analyzed expression patterns of eight randomly selected *BdMYB* genes from different subfamilies by quantitative real-time PCR (qRT-PCR). The results showed that six genes were expressed in all organs. In particular, seven *BdMYB* genes are expressed in flowers at a high level (**Fig. 7**). We also analyzed the expression of these genes under different abiotic stresses in 2-week old seedlings. The results showed that the expression of seven genes was up-regulated in ABA-treated seedling roots (**Fig. 8A**). In leaves, the expression of *BdMYB056* and *BdMYB091* was drastically down-regulated under different stresses (**Fig. 8B**).

Discussion

Duplication contributed to the *BdMYB* gene expansion

Gene duplication is a crucial origin to generate new genes (Davidson et al. 2013). It contributes significantly to the proliferation of *MYB* genes in the plant kingdom (Hou et al. 2014), and leads to gene diversification or drives the evolution of genes. Our results showed that 26 (21.31%) *BdMYB* genes were identified as tandem duplication genes and 55 (45.08%) *BdMYB* genes were found to be located as segmental repeats in *B. distachyon*, indicating that tandem and segmental duplication events were vital causes of the expansion of *BdMYB* genes.

Quite a few studies indicated that the plant *MYB-R1R2R3* genes originated from *MYB-R2R3* genes by obtaining the R1 repeat through an ancient intragenic duplication or forming a MYB-related gene through duplication of the R1 repeat (Rosinski and Atchley 1998, Jiang et al. 2004), and it was proposed that the duplication of R2 in an early form gave rise to the MYB-R1R2R3 domains (Rosinski and Atchley 1998). For example, the MYB-related gene *BdMYB035* may be derived

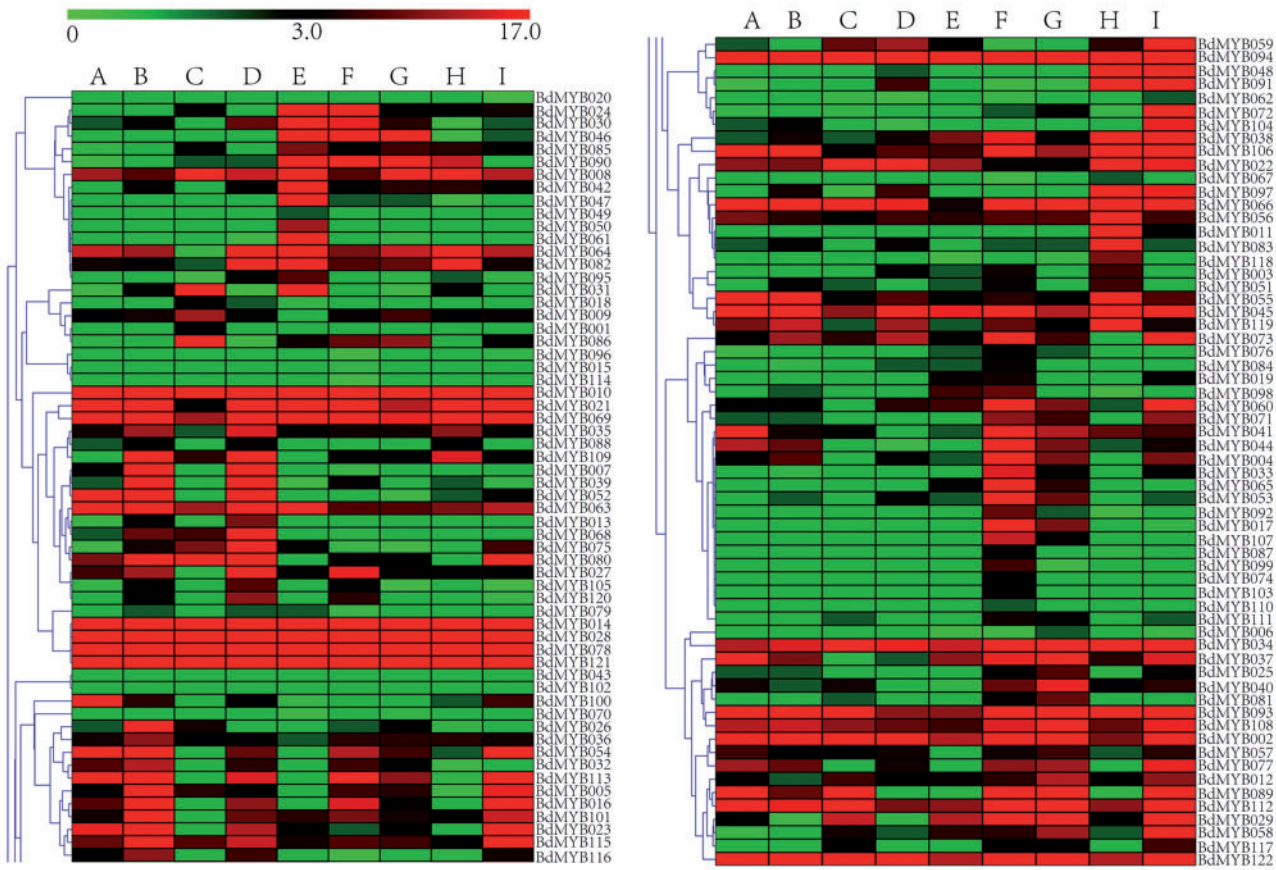

Fig. 5 The expression profiles of *BdMYB* genes in different tissues based on the transcriptome data. These data were used to analyze the expression profiles of *BdMYB* genes in nine different tissues, i.e. seeds 5 d after pollination (A), seeds 10 d after pollination (B), plant embryo (C), endosperm (D), leaves (E), emerging inflorescences (F), early inflorescences (G), anthers (H) and carpels (I). The left panel is the upper part of the figure and the right panel is the bottom part.

from the *MYB-R1R2R3* gene *BdMYB056* with loss of two R repeats. Most of the gene duplications resulted from segmental duplications, and a large number of *MYB-R2R3* genes originated from segmental duplication events. As a result, the *B. distachyon* genome has more *MYB-R2R3* genes.

Duplicated genes often evolve to lose the original functions and/or to obtain new functions to enhance the adaptability of plants (Dias et al. 2003). Previous research demonstrated that a diversified expression pattern and response to various abiotic stresses might be a significant reason for retaining duplicated genes in the genome (Gu et al. 2002). We found that pairs of *BdMYB* genes displayed a different expression level in different tissues and under abiotic stresses in this study. For example, in one pair, *BdMYB075* was expressed at a high level while *BdMYB076* was expressed at a low level. Further analysis showed that *BdMYB075* had *cis*-elements such as a TCA element, an MBS and HSE in response to drought and heat stress in the promoter regions. In contrast, they were not found in the promoter region of *BdMYB076*.

The *BdMYB* genes play important roles in flower development

When plants transit from the vegetative stage to the reproductive stage, the expression of a large number of *MYB* genes is required (Clavijo Michelangeli et al. 2013). Previous analysis demonstrated that many *MYB* genes were expressed in flowers. For example, *AtMYB17* is expressed in inflorescences and siliques at early flower developmental stages (Zhang et al. 2009); *AtMYB118* is expressed predominantly in siliques (Zhang et al. 2009). Similarly, *AtMYB33* shows a predominant floral expression pattern (Gocal et al. 2001). In rice, *OsMYB511* is mainly expressed in panicles (Huang et al. 2015), and *OsMPS* is expressed in vegetative and reproductive tissues (Schmidt et al. 2013). In *B. distachyon*, *BdMYB055*, the close homolog of *AtMYB33*, *BdMYB011* and *BdMYB083* are highly expressed in anthers, while *BdMYB089* is expressed particularly in the emerging inflorescence (Davidson et al. 2012). In this study, we found that 15.57% (19/122) of *MYB* genes were highly expressed in the inflorescence (**Fig. 5**). Among eight selected *BdMYB* genes, seven genes are expressed in flowers at a high level (**Fig. 7**). These results prompted us to predict that *MYB* genes might play important roles in flower development.

Indeed, many *MYB* genes were reported to be involved in flower development. In Arabidopsis, *MYB21*, *MYB24*, *MYB33*,*MYB57*, *MYB65* and *MYB103* are regulators of several pathways to affect anther development (Li et al. 1999, Millar and Gubler 2005, Cheng et al. 2009, Zhu et al. 2010, Reeves et al. 2012). Meanwhile, *MYB21* and *MYB24* promote the

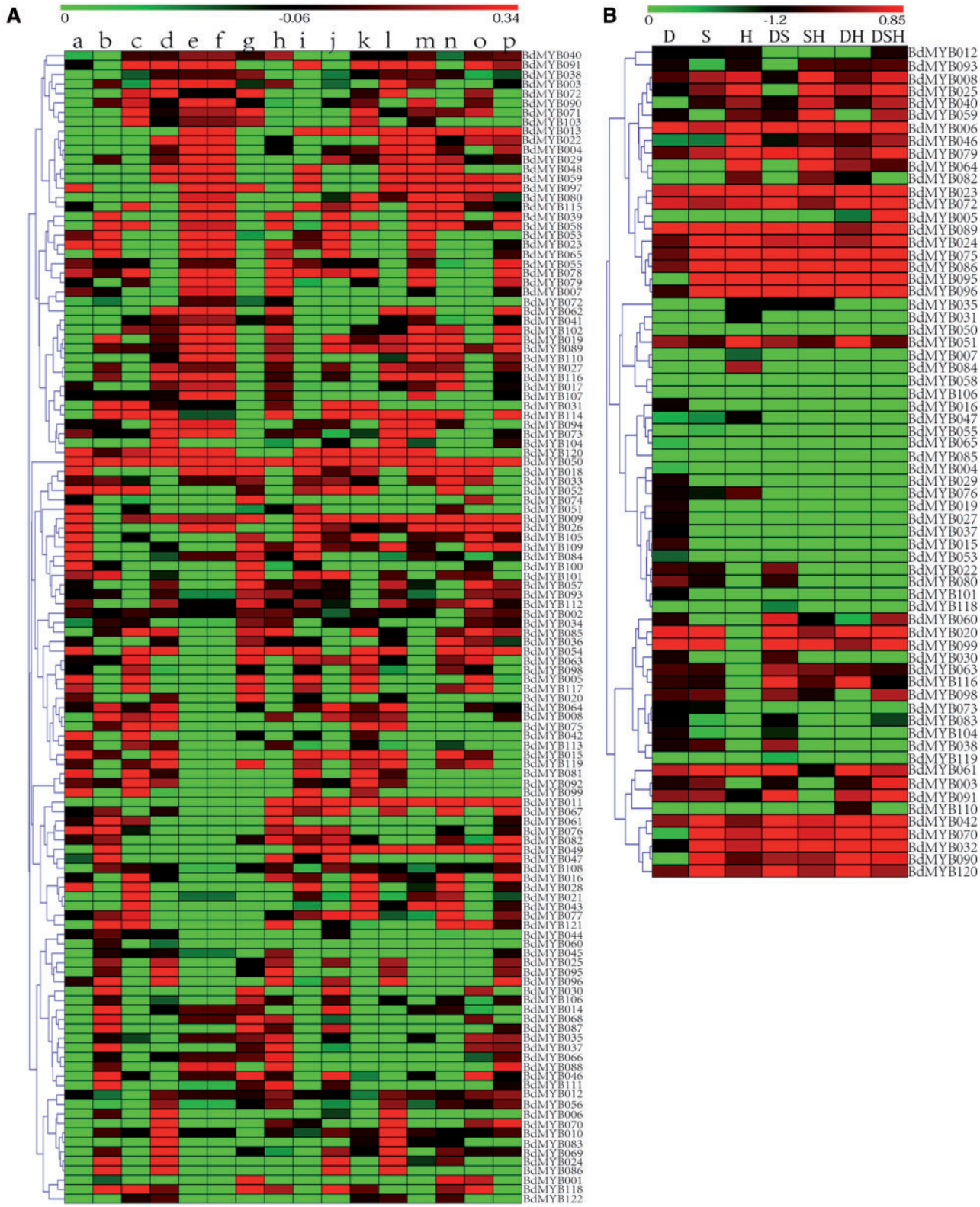

Fig. 6 The expression profiles of *BdMYB* genes under (A) hormone stress and (B) other abiotic stresses based on the transcriptome data. The expression profiles of *BdMYB* genes under different abiotic stresses. (A) *BdMYB* genes under hormone stresses, including a/i (auxin), b/j (cytokinin), c/k (salicylic acid), d/l (ABA), e/m (jasmonate), f/n (prohexadione), g/o (brassinosteroid) and h/p (ethylene) in high (a, b, c, d, e, f, g, h) and low (i, j, k, l, m, n, o, p) stringency conditions. (B) Expression profiles of *BdMYB* genes under abiotic stresses, including D (drought), S (single salinity), H (heat), DS (drought and salinity combination), SH (salinity and heat combination), DH (drought and heat combination) and DSH (drought, salinity and heat combination).

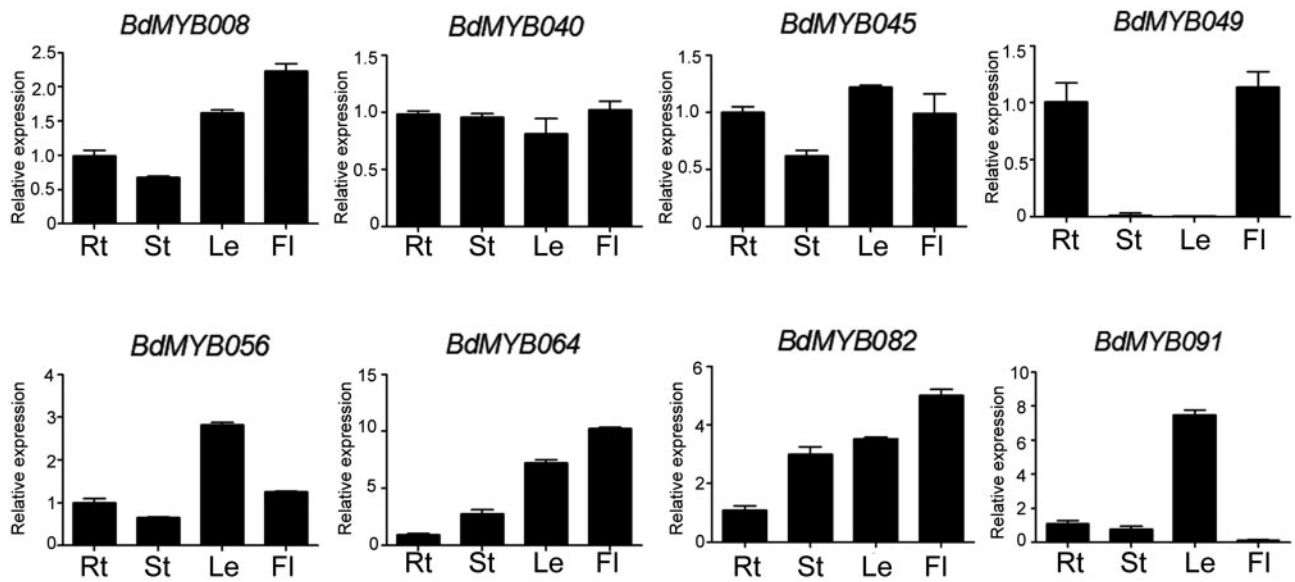

Fig. 7 Results of quantitative RT-PCR of eight *BdMYB* genes in different tissues. Rt (roots), St (stems), Le (leaves) and Fl (inflorescences) were collected at the heading stage (8 weeks old). The horizontal and vertical co-ordinates stand for four different tissues and the relative expression, respectively.

development of petals and gynoeca (Reeves et al. 2012). *AtMYB32* is necessary for normal pollen development and *MYB98* is required for the guidance of the pollen tube and cell differentiation of synergids (Preston et al. 2004, Kasahara et al. 2005). Additionally, the disruption of *AtMYB26* results in male sterility (Steiner-Lange et al. 2003). In rice, two *MYB* genes, *AID1* and *CSA*, function in anther development (Zhu et al. 2004, Zhang et al. 2010). In *B. distachyon*, however, there has been no report to prove *MYB* genes regulate flower development directly; the expression pattern indicates that some *MYB* genes might regulate flower development. Taken together, the expression pattern and previous studies suggested that *MYB* genes play an important role in flower development.

MYB TFs function in response to abiotic stresses

Many MYB TFs function in response to abiotic stresses. For example, overexpression of two wheat *MYB* genes, *TaMYB73* and *TaMYB33*, improved salt stress tolerance in Arabidopsis (He et al. 2012. Qin et al. 2012). Similarly, overexpression of *AtMYB44* conferred enhanced tolerance to multiple abiotic stresses in Arabidopsis (Jung et al. 2008). Some MYB TFs participate in abiotic stresses by modulating the expression of target genes. For example, TaMYB19-B altered the expression level of many abiotic stress-related genes that overcome adverse conditions (Zhang et al. 2014); TaMYB73 participated in salinity tolerance via the regulation of a number of stress-responsive genes (He et al. 2012).

The expression profiles of many *MYB* genes indicate their probable functions in response to abiotic stresses. For example, in rice, the expression of *OsMYB511* and *OsMYB2* is markedly induced by cold (Yang et al. 2012, Huang et al. 2015); the expression of *OsMYB511* and *CMYB1* is dramatically induced by osmotic stress and exogenous ABA in rice (Duan et al. 2014, Huang et al. 2015). In Arabidopsis, the expression of *MYB21*/*At3g27810* and *MYB24*/*At5g40350* is rapidly induced by jasmonate (Stracke et al. 2001). Additionally, the expression of *TaMYB4* and its homologous gene *BdMYB078* is induced by the hormones SA, ethylene, ABA and MeJA (Al-Attala et al. 2014); the expression of *OsMYB091* and its homologous gene *BdMYB089* is up-regulated by SA, ABA, MeJA and gibberellin (Zhu et al. 2015). Like in Arabidopsis, wheat and rice, the expression of many *BdMYB* genes is also induced by abiotic stress. For example, the expression of *BdMYB091* and *BdMYB115* is up-regulated by heat stress (Chen and Li 2016), while the expression of *BdMYB078* is induced by cold and wounding treatments (Al-Attala et al. 2014). In this study, the results showed that the expression of eight randomly selected *BdMYB* genes is regulated by heat, drought and salinity stress in roots and leaves of seedlings and the expression of seven *BdMYB* genes is up-regulated by ABA in roots. A previous study demonstrated that the ABRE is the major *cis*-element for ABA-responsive gene expression (Yamaguchi-Shinozaki and Shinozaki 2006). We found that 72.13% (88/122) of *BdMYB* genes had ABREs. These results further suggest the probable function of *BdMYB* genes in response to abiotic stresses.

Materials and Methods

Identification, sequence alignment and phylogenetic analyses of *MYB* genes in *B. distachyon*

In order to identify the *MYB* genes in *B. distachyon*, the HMM profile of the Myb_DNA-binding domain (PF00249) was obtained in Pfam v30.0 (http://pfam.xfam.org/) (Finn et al. 2016) and searched against the protein sequence of *B. distachyon* with a threshold of e < 1e-5. The MYB protein sequences of 197 Arabidopsis and 155 rice TFs were retrieved from the Ensembl Plant database (http://plants.ensembl.org/index.html), then the BLASTP program with the threshold of e < 1e-5 and identity of 50% was used to search against the

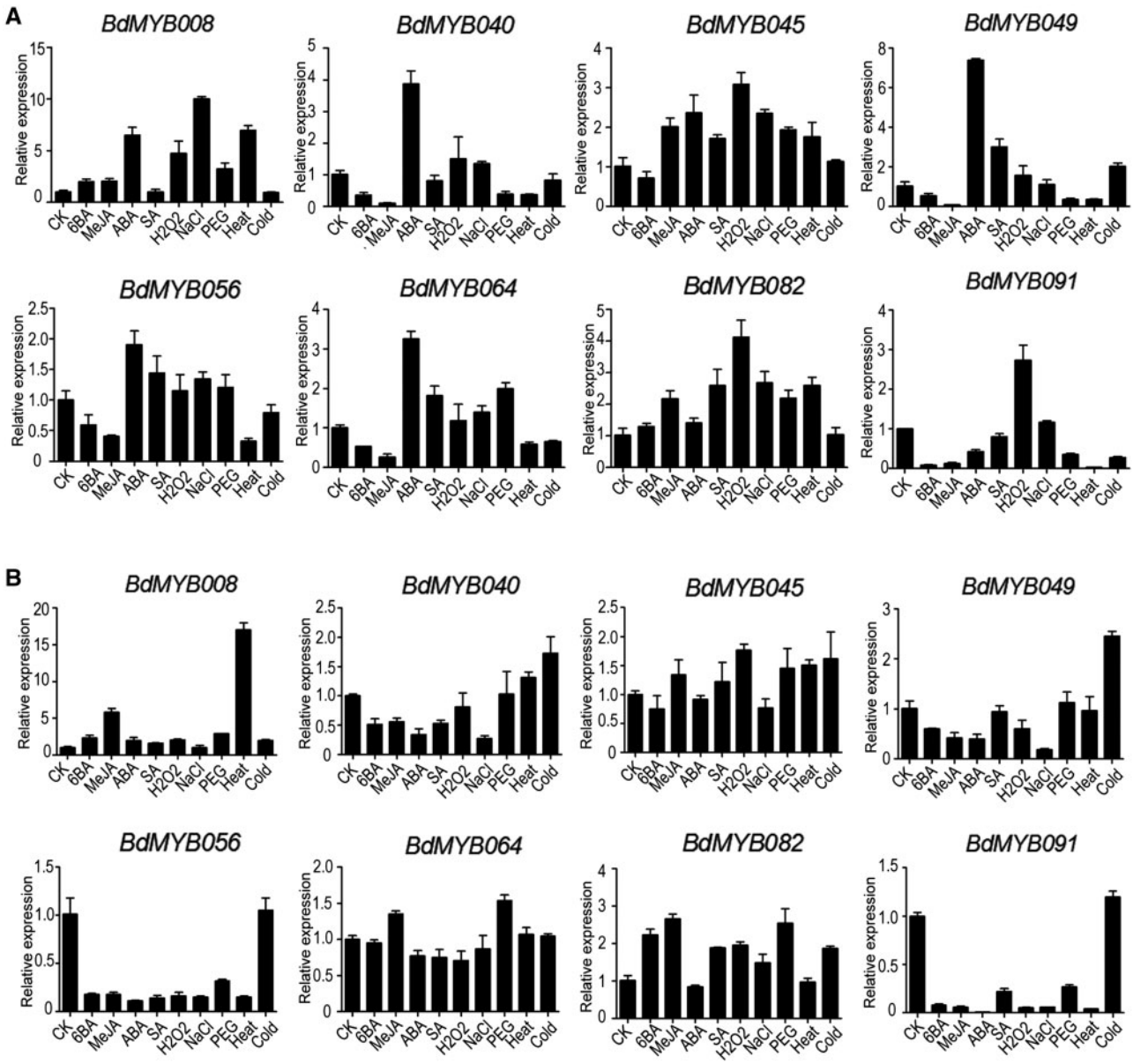

Fig. 8 Quantitative RT-PCR expression analyses of eight *BdMYB* genes of (A) seedling roots and (B) leaves under different abiotic stresses. Under abiotic stresses, 2-week-old seedlings were subjected to 6-benzyladenine (20 μM), MeJA (100 μM), ABA (100 μM), SA (100 μM), H_2O_2 (10 mM), NaCl (200 mM), drought (20% PEG6000), heat (42°C) and cold (4°C) for 2 h. The horizontal and vertical axes stand for different treatments and the relative expression level, respectively.

B. distachyon proteins. After BLASTP, a self-blast and manual correction was performed to remove the alternative splicing events and any redundancy. Finally, the NCBI-CDD web server (https://www.ncbi.nlm.nih.gov/cdd/) (Marchlerbauer et al. 2015) and SMART (http://smart.embl-heidelberg.de/) (Letunic et al. 2015) were used to confirm the obtained BdMYB proteins.

The unrooted NJ tree was constructed with 1,000 bootstrap replications using MEGA7 (Kumar et al. 2016) based on the full-length protein alignment. The protein sequences, cDNA sequences, DNA sequences, upstream 1.5 kb genomic DNA sequences and coding sequences of BdMYB TFs were downloaded from the Ensembl Plants database (http://plants.ensembl.org/index.html) for further analysis. CELLO v.2.5 (http://cello.life.nctu.edu.tw/) (Yu et al. 2004) was used to predict the subcellular location of BdMYB TFs. The theoretical isoelectric point and molecular weight of the BdMYB TFs were predicted by the ProtParam tool (http://web.expasy.org/protparam/) (Wilkins et al. 1999).

Analyses of gene structure and conserved motifs

The exon–intron structure of these genes was graphically displayed by the Gene Structure Display Server (Hu et al. 2015) using the CDS and genome sequence of *BdMYB* genes. The protein sequence of BdMYB TFs was used to predict the conserved motifs by using the MEME Suite web server (http://meme-suite.org/) (Bailey et al. 2009) with the maximum number of motif sets at 10 and optimum width of motifs from five to 200 amino acids.

Analyses of stress-related *cis*-elements and GO annotation

The upstream 1.5 kb genomic DNA sequences of *BdMYB* genes were submitted to the Plant CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Rombauts et al. 1999) to identify the *cis*-elements in the promoters. The GO (http://www.geneontology.org/) (Ashburner et al. 2000)

annotation of BdMYB proteins was submitted to the EMBL-EBI Inter Pro tool (Finn et al. 2017) and the Plant Transcriptional Regulatory Map (Jin et al. 2015) to predict the functions of BdMYB proteins. Then the annotation was visualized and plotted by BGI WEGO (Ye et al. 2006).

Analyses of chromosome distribution, gene duplication and synteny

The chromosome distribution information of *BdMYB* genes was obtained from the Ensembl Plant database (http://plants.ensembl.org/index.html). The gene duplication and synteny information was analyzed using a previously reported method (Cui et al. 2016). Segmental duplication, tandem duplication and duplications between *B. distachyon* genes as well as the synteny block of MYB between *B. distachyon* and rice, maize and sorghum were obtained from the Plant Genome Duplication database (http://chibba.pgml.uga.edu/duplication/) (Lee et al. 2013) and visualized (including gene locations) using the Circos v0.55 (Krzywinski et al. 2009). To estimate duplication events of *BdMYB* genes further, the Ks and Ka of evolution were calculated by using DnaSP v5 (Librado and Rozas 2009). The divergence times (T) was calculated as $T = Ks/2\lambda \times 10^{-6}$ Mya; the approximate value for the clock-like rate $\lambda = 6.5 \times 10^{-9}$ for *B. distachyon* (Lynch and Conery 2000).

Prediction of BdMYB protein–protein interaction network

On the basis of the Arabidopsis orthologous proteins, the interactions between BdMYB proteins and other proteins were predicted by using the online program STRING 10 (https://string-db.org/) with the option value > 0.800 (Szklarczyk et al. 2017). Then the homologs of these interaction proteins in *B. distachyon* were identified using the best hits of BLASTP analysis with a threshold of e-value < 1e-5. The interaction network was visualized by Cytoscape v3.4.0 (Shannon et al. 2003).

Analysis of gene expression profiles

High-throughput sequencing data of *B. distachyon* were obtained from the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) (Parkinson et al. 2007) under accession number E-MTAB-4401, and in the DDBJ Sequence Read Archive database (http://www.ddbj.nig.ac.jp) (Mashima and Kodama 2017) under accession numbers PRJDB2997 and PRJNA360513. These data were used to analyze the expression profiles of *BdMYB* genes in nine different tissues (i.e. seed 5 d after pollination, seed 10 d after pollination, plant embryo, endosperm, leaf, emerging inflorescence, early inflorescence, anther and carpel) and under hormone stresses (auxin, cytokinin, SA, ABA, MeJA, prohexadione, brassinosteroid and ethylene), as well as other abiotic stresses (single salinity, drought and heat stress as well as their double and triple stress combinations).

Plant growth and stress treatment

Brachypodium distachyon Bd-21 was planted in an artificial climate chamber at 26/22°C (day/night) with a photoperiod of 16/8 h (day/night). For different tissue analyses, roots, stems, leaves and inflorescences were collected at the heading stage (8 weeks old). For abiotic stress, 2-week-old seedlings were subjected to 6-benzyladenine (20 μM), MeJA (100 μM), ABA (100 μM), SA (100 μM), H_2O_2 (10 mM), NaCl (200 mM), drought (20% PEG6000), heat (42°C) and cold (4°C) for 2 h, and then the leaves and roots were collected for RNA isolation.

RNA extraction, cDNA synthesis, and quantitative RT-PCR

RNA extraction and cDNA synthesis were carried out as described previously (Chen and Li 2016, Liu et al. 2016). The qRT-PCRs were performed in triplicate and 15 μl reaction systems containing 7.5 μl of SYBR® Premix Ex Taq (TAKARA), 0.75 μl (10 pmol μl^{-1}) each of forward and reverse primers, 0.5 μl of cDNA (5.0 ng μl^{-1}) and 5.5 μl of ddH_2O. The qRT-PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min in the PCR stage and 95°C for 15 s, 60°C for 1 min, 95°C for 15 s in the melt curve stage. Data acquisition and analyses were performed using the QuantStudio™ Real-Time PCR Software (ThermoFisher Scientific). Data were normalized to the *ACTIN* gene (Hong et al. 2008) and the relative expression level was calculated using the $2(-\Delta\Delta Ct)$ analysis method (Livak and Schmittgen 2001). The primers used in this study are listed in **Supplementary Table S9**.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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