Deciphering the roles of tobacco MYB transcription factors in environmental stress tolerance

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The MYB members play important roles in development, metabolism, and stress tolerance in plants. In the current study, a total of 246 tobacco R2R3-MYB transcription factors were identified and systemically analyzed from the latest genome annotation. The newly identified tobacco members were divided into 33 subgroups together with the Arabidopsis members. Furthermore, 44 NtMYB gene pairs were identified to arise from duplication events, which might lead to the expansion of tobacco MYB genes. The expression patterns were revealed by transcriptomic analysis. Notably, the results from phylogenetic analysis, synthetic analysis, and expression analysis were integrated to predict the potential functions of these members. Particularly, NtMYB102 was found to act as the homolog of AtMYB70 and significantly induced by drought and salt treatments. The further assays revealed that NtMYB102 had transcriptional activities, and the overexpression of the encoding gene enhanced the drought and salt stress tolerance in transgenic tobacco. The results of this study may be relevant for future functional analyses of the MYB genes in tobacco.

KEYWORDS
MYB transcription factors, tobacco, metabolism, stress tolerance, NtMYB102

Introduction

Transcription factor is the important regulator that controls the expression of target genes, and they play important roles in plant development, metabolism, and stress response (Dubos et al., 2010; Katiyar et al., 2012; Colet et al., 2021). The MYB transcription factor family is the one of largest transcription factor families in plants...
et al., 1997; Stracke et al., 2001). Generally, each MYB member contains a highly conserved DNA binding domain, composed of 50–52 amino acids, which binds the DNA in the form of a helix-turn-helix structure (Moyano et al., 1996). According to the repeat number of MYB domains, the MYB family members could be divided into four groups, including 4R-MYB, R1R2R3-MYB, 1R-MYB, and R2R3-MYB, and the R2R3-MYB members are the most common subgroup in plants (Stracke et al., 2001; Dubos et al., 2010). Interestingly, a conjecture is that R2R3-MYB proteins have likely evolved from R1R2R3-MYB precursors by the loss of the R1 repeat. On the contrary, the R1R2R3-MYB might evolve from R2R3-MYB by acquiring an R1 repeat. (Stracke et al., 2001; Jiang et al., 2004; Dubos et al., 2010). Since the first plant MYB gene was cloned from maize (Paz-Ares et al., 1987), a variety of MYB transcription factors in plants have been identified and analyzed (De Vos et al., 2006; Zou et al., 2013; Stracke et al., 2014; Qiu et al., 2019; Chen et al., 2021; Naik et al., 2021; Qin et al., 2021).

In plants, the R2R3-MYB subfamily members have been found to participate in various biological processes including plant development, secondary metabolism, and biotic/abiotic stress responses (Stracke et al., 2001; Castillejo et al., 2020; Cao et al., 2021, Ding et al., 2021). In Arabidopsis, 126 R2R3-MYB transcription factors have been divided into 25 subgroups, and a number of them had been reported to play an important role in primary and secondary metabolism (Allan et al., 2008; Dubos et al., 2010; Roy, 2016). In subgroup 7, as the typical MYB transcription factors, AtMYB11/PFG2, AtMYB12/PFG1, and AtMYB111/PFG3 were reported to be involved in the accumulation of specific flavonol derivatives in leaves, stems, inflorescences, siliques, and roots (Moyano et al., 1996; Stracke et al., 2007). In subgroup 6, the AtMYB75/PAP1 and AtMYB90/PAP2 transcription factors regulate the biosynthesis of anthocyanins in vegetative tissues (Quattrocchio et al., 1999; Appelhagen et al., 2011). Overexpression of AtMYB113 or AtMYB114 leads to a significant increase in anthocyanins production (Gonzalez et al., 2008). In addition, multiple R2R3-MYB genes have been reported to be involved in the synthesis of Arabidopsis secondary cell walls (Zhao and Dixon, 2011). In subgroup 3, AtMYB58 and AtMYB63 affect the SND1-mediated (secondary wall-associated NAC domain protein 1) transcription network regulating secondary wall formation (Zhou et al., 2009). In subgroup 16, AtMYB83 and AtMYB46 are both direct targets of the SND1 transcription factor and play the redundant role in the transcriptional regulatory cascade, which allows plants to form secondary walls by regulating fibers and blood vessels (Mccarthy et al., 2009).

Some R2R3-MYB genes have also been reported to participate in the growth and development of plants (Stracke et al., 2001; Li et al., 2020; Pucker et al., 2020). AtMYB77 of subgroup 22 was reported to control lateral root growth and development (Shin et al., 2007). In subgroup 14, AtMYB68 specifically regulates root growth, influencing the whole plant development under harsh conditions (Feng et al., 2004). AtMYB59 was reported to regulate the development of roots by regulating the cell cycle of the root tip (Mu et al., 2009). In flower development, AtMYB33 and AtMYB65, which belong to subgroup 18, facilitate both anther and pollen development, and the pollen fails to maintain vitality in myb33/myb65 (Millar and Gubler, 2005). AtMYB120 is studied to be a pollen-specific factor, which controls the pollination of plants and the differentiation and development of pollen (Jiang et al., 2013). In subgroup 21, AtMYB105/LOF2 and AtMYB117/LOF1 control the separation of the lateral stem of the plant, and AtMYB91/AS1 participates in the regulation of leaf patterning (Byrne et al., 2000; Lee et al., 2009). In subgroup 20, AtMYB2 is reported to regulate leaf senescence, and in subgroup 22, AtMYB44/MYBR1 was involved in the regulatory network of leaf senescence and ABA signaling (Guo and Gan, 2011; Jaradat et al., 2013).

Unlike animals, plants grow in a complex environment and face multiple biotic/abiotic stresses from the environment; some R2R3-MYB transcription factors have been confirmed to respond to the stresses in plants (Stracke et al., 2001; Dubos et al., 2010). For instance, AtMYB60 and AtMYB96 of subgroup 1 are reported to improve the plant drought resistance by regulating the closure of plant stomata (Seo et al., 2009; Oh et al., 2011). Similarly, in subgroup 22, AtMYB70, AtMYB73, and AtMYB74 also regulate stomatal closure to improve plants' anti-stress ability (Jung et al., 2008). In addition, several R2R3-MYB members are also involved in cold, salinity, and wounding stresses. In subgroup 2, AtMYB2 is reported to increase plant salt resistance through ABA signaling, while AtMYB15 is involved in the regulation of cold tolerance; myb15 increased plants' tolerance to cold stress whereas its overexpression reduced plants' cold tolerance (Abe et al., 2003; Agarwal et al., 2006). In defense response, AtMYB102 keeps plants from being damaged by herbivore Pieris rapae. AtMYB41 is reported to be involved in the negative regulation of short-term transcriptional response to osmotic pressure, and AtMYB72 is essential for Arabidopsis to fight against various fungal and bacterial diseases (De Vos et al., 2006; Cominelli et al., 2008; Van Der Ent et al., 2008; Segarra et al., 2009). Besides, in subgroup 22, overexpression of AtMYB44 could promote Botrytis infection, and AtMYB44 positively regulates the disease resistance of Pseudomonas syringae in Arabidopsis through the salicylic acid signaling pathway (Jung et al., 2008).

Tobacco is not only a considerably economically valuable crop but also a well-studied model organism. However, extreme environment and diseases have always been the potential threat to tobacco yield and quality. Therefore, the study for systematic analysis of the MYB transcription factor family in tobacco is of great significance for the research of tobacco secondary metabolism, growth development, and resistance to biotic/abiotic stresses. With the publication of multiple plant genome sequences, a new insight combining bioinformatics and
molecular biology began to identify and analyze the R2R3-MYB transcription factor family and their functions. So far, the function of the R2R3-MYB protein has been discovered and verified in a large number of plants, such as Arabidopsis (Dubos et al., 2010), tomato (Solanum lycopersicum) (Li et al., 2016), potato (Solanum tuberosum) (Li et al., 2019), Chinese pear (Pyrus bretschneideri Rehd) (Cao et al., 2016), maize (Zea mays) (Du et al., 2012), wheat (Triticum aestivum) (Wei et al., 2020), and rice (Oryza sativa) (Katiyar et al., 2012). However, very limited research is available on tobacco R2R3-MYB members. Here, we identified 246 R2R3-MYB members from the tobacco genome sequences using comparative genomic and molecular biology methods, then inferred the function of R2R3-MYB protein from phylogenetic trees, collinearity, and expression patterns. This study will provide a solid foundation for further functional studies on the function of R2R3-MYB members in tobacco growth, metabolism, and biotic/abiotic stresses.

Materials and methods

Identification and classification analysis of tobacco R2R3-MYB members

Version 4.5 of the genome sequence annotations of tobacco (Nicotiana tabacum L.) were downloaded from the SGN database (https://solgenomics.net/). The previously reported AtMYB full-length protein sequences (Stracke et al., 2001; Dubos et al., 2010) were obtained from The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/) and used as queries to perform the BLASTP search against the annotated tobacco protein databases with an E-value cutoff of 0.01. Furthermore, with the HMM profile (PF00249), the HMM search was performed against the annotated tobacco protein databases under the E-value cutoff of 0.001. The candidate sequences from the two above-described approaches were integrated, and redundant entries were removed manually. The putative MYB protein sequences were analyzed using both Pfam (https://pfam.xfam.org/) with an E-value cutoff of 1.0 and SMART (http://smart.embl.de/) with an E-value cutoff of 1.0 to detect the presence and number of the MYB domain (Letunic et al., 2020; Mistry et al., 2021).

The full-length protein sequences of Arabidopsis R2R3-MYB members and newly identified tobacco R2R3-MYB members were subjected to the performance of multiple sequence alignment using MAFFT v5.3 under the default settings (Katoh and Standley, 2013). The alignment of R2R3-MYB domain was visualized using TBtools (Chen et al., 2020). Subsequently, the neighbor-joining phylogenetic analysis was conducted using MAGE X based on the alignment of full-length protein sequences with a bootstrap method of 1000 replicates, substitution with the Poisson model, and pairwise deletion (Kumar et al., 2018). The phylogenetic tree was displayed using FigTree v1.3.

Exon–intron structural analysis and identification of conserved motifs

The genomic sequences and coding sequences of AtMYB and NtMYB genes were submitted to the Gene Structure Display Server (Hu et al., 2015) to visualize their exon–intron structure. Further, the MEME Suite 5.1.1 tool (Bailey et al., 2015) was hired to explore the conserved motifs of the AtMYB and NtMYB proteins with following parameters: maximum number of motifs: 10; the optimum width of each motif: between 6 and 100 residues.

Chromosomal localization and duplication event analysis

According to the location information provided by the Solanaceae database, the Perl program was adopted to display the NtMYB genes’ chromosomal location. The tandem gene events were displayed on the chromosomal map according to the previous definition (Li et al., 2018). Afterward, the TBtools’ Circos program (Chen et al., 2020) was recruited to analyze the synteny relationship of the orthologous genes from tobacco and five other species (including Arabidopsis, tomato, potato, maize, and rice).

Promoter analysis of tobacco R2R3-MYB genes

The sequences 2000 bp upstream of the R2R3-MYB genes in tobacco were extracted from the genome sequence database. The obtained sequences were subjected to PlantCARE platform analysis to further search for the putative cis-elements in their promoter regions (Lescot et al., 2002).

Expression patterns analysis

The reported RNA-seq data of tobacco tissues (Edwards et al., 2017) were downloaded from the GEO database (accession number: GSE95717). The processed expression data of the R2R3-MYB genes were extracted (Supplementary Table S1) and transformed with log2 to normalize the raw data using TBtools (Chen et al., 2020).
The RNA-Seq data of the tobacco senescent leaf were produced in our previous work (Li and Guo, 2018) and were uploaded at the NCBI Short Read Archive (SRA) under the accession number SRP102153. The plants were grown with regular practices and topping was done 60 days after transplanting. The middle leaves were collected at nine different time points and used for transcriptomic analysis. The expression data of the R2R3-MYB genes were retrieved (Supplementary Table S2) and normalized by comparing with 5 DAT (days after topping) and log2 fold change transformation. All the normalized RNA-seq data were used to illustrate Heatmap using the pHeatmap R package under the default parameters.

**Tobacco plant preparation and stress treatments**

Cultivated tobacco K326 was used to analyze the expression pattern of NtMYB in this study. The roots, stems, flowers, upper leaves, middle leaves, and lower leaves were collected and frozen in liquid nitrogen when cultivated tobacco K326 grew to the budding stage; the samples were stored in a refrigerator at -80°C for later use.

For the drought stress treatment assays, T3 transgenic and wild-type (K326) tobacco seeds were sown into the soil and treated after about 6 weeks as with previous conditions (Li et al., 2022). The leaves of the seedlings were detached and air-dried, then the leaf weight was recorded at 180 min.

For the salt stress treatment assays, the tobacco seedlings were germinated in a solid MS medium after disinfection, then they were transferred to a liquid MS medium and adapted for 5 days. Hereafter, some of the seedlings were transferred to a 150 mmol/l NaCl liquid MS medium or absorbent filter paper for salt and drought treatment, respectively. Samples were taken at 1, 3, and 6 h after treatment and frozen in liquid nitrogen for later use. Three biological replicates were performed for each sample.

**RNA Extraction and qTR-PCR**

Total RNAs from each sample were extracted using the Ultrapure RNA Kit (cwbiotech, Beijing, China), then the first-strand complementary DNA (cDNA) was synthesized using the Evo M-MLV Mix Kit with gDNA Clean for qPCR (Accurate Biotechnology, Changsha, China). Quantitative real-time PCR (qRT-PCR) reactions were performed in a Roche LightCycler 480 Real-Time PCR instrument with SYBR® Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology, Changsha, China). The tobacco ribosomal protein gene L25 (GenBank No. L18908) was used as control (Li et al., 2021). All experimental data were obtained through three technical repetitions and three biological replicates; the relative expression level was calculated by the \( \triangle \triangle CT \) method (Livak and Schmittgen, 2001). The details of the primers are provided in Supplementary Table S3.

**Subcellular localization**

The CDS of the NtMYB102 gene was amplified from the cDNA of tobacco root using Phanta® Max Master Mix (Vazyme, Nanjing, China) and ligated to the pEasy-Blunt Mix (Accurate Biotechnology, Changsha, China) for later use; then the sequence of the NtMYB102 encoding gene without stop codon was inserted into the PYG57 vector, which was started by the CaMV-35S promoter and contained the GFP fragment (Sun et al., 2021). The construct was transformed into an Agrobacterium competent cell GV3101, and transiently expressed in the leaves of Nicotiana benthamiana. Simultaneously, the empty vector injected leaves as a control. Three days after the injection, the leaves were soaked in DAPI staining solution to determine the location of the nucleus, as previously reported (Li et al., 2019). Fluorescence signals were captured using a Confocal Microscope (TCS-SP8 Leica, Wetzlar, Germany).

**Transcriptional activation assay**

The CDS of NtMYB102 was amplified and inserted into the EcoR I site of the pBridge vector, using an Infusion HD Cloning Kit (Takara, Shiga, Japan) to be fused with a GAL4 DNA binding domain. The construct and the control vector were introduced into the yeast strain AH109 separately, followed by growing yeasts on SD/-Trp, and SD/-Trp supplemented with 5-bromo-4-chloro-3-indolyl-a-d-galactopyranoside (X-a-Gal) for 4 days at 30°C. The transcriptional activation activities were evaluated based on the growth status of different transformants.

**Tobacco transgenic plant and root length analysis**

The coding sequence of the NtMYB102 gene was amplified from the cDNA and inserted into the pCHF3 vector, which was driven by the CaMV-35S promoter, to complete the construction of the overexpression vector. The pCHF3 plasmid containing the NtMYB102 gene was transformed using the Agrobacterium-mediated method (Buschmann, 2016). To obtain T1 generation homozygous lines, the seeds of several T0 independent lines were selected on 50 mg/l kanamycin MS medium with no separation. The homozygous T1 lines and wild-type (K326) seeds were sterilized and grown in a vertical MS medium for 14 days, then transferred to an MS medium with 0 or 100 mM NaCl, respectively, to observe changes in root.
length. The significant difference analysis was calculated using SPSS v18.0 with the \( t \)-test.

**Results**

**Identification of R2R3-MYB members in tobacco**

To identify the R2R3-MYB members in tobacco, the BLASTP and HMMER searches were performed using the previous R2R3-MYB protein sequences from *Arabidopsis* as the query. Eventually, a total of 246 MYB members were obtained from tobacco genome sequences. Among them, 145 NtMYB genes distribute unevenly on the tobacco chromosomes, while the others map on the scaffolds. To distinguish the newly identified genes, theses R2R3-MYB genes were named according to physical order on the chromosome and scaffolds. The detailed information could be explored in Supplementary Table S4.

**Multiple sequence alignment and phylogenetic analysis**

In plants, the DNA binding domain with two adjacent MYB repeats is conserved in R2R3-MYB members (Jiang et al., 2004). To explore the features of the R2R3-MYB members of tobacco, the newly identified R2R3-MYB domain performed multiple sequence alignments. As the result, the tobacco R2 and R3 MYB repeats hold the conserved amino acid residues. Notably, the tobacco R2R3-MYB domain features were found to be highly similar with *Arabidopsis* members (Supplementary Figure 1A). In the R2 MYB repeat, three Tryptophan (W) residues were found to be conserved, while only two Tryptophan (W) residues were found to be conserved in the tobacco R3 MYB repeat; the first Tryptophan (W) was replaced by Phenylalanine (F) or others (Supplementary Figure 1B).

As a result, all of the R2R3-MYB members were divided into 33 subgroups, among them, the S1 to S25 subgroups were consistent with the previous reports (Dubos et al., 2010); the others were named from S26 to S33, which contained some tobacco and *Arabidopsis* R2R3-MYB members (Figure 1). Results showed that most of the subgroups contained R2R3-MYB members from those two species, indicating that the expansion of R2R3-MYB members may appear before the divergence of tobacco and *Arabidopsis*. Interestingly, several subgroups contained much more R2R3-MYB members from tobacco than *Arabidopsis*, such as S1, S2, and S14, implying that the duplication events might occur in those subgroups. Notably, it was found that S17, S29, S31, and S32 only harbored R2R3-MYB members from tobacco and S12 only contained members from *Arabidopsis*.

**Gene structure and conserved, motif analysis**

The gene structure could provide the clues of a gene family evolution history. As a result, the intron number of studied genes was found to range from 0 to 11, and NtMYB genes shared the similar gene structures with *Arabidopsis* MYB genes in the same group (Supplementary Figure S2). Interestingly, most (82.1%) of the coding sequences of NtMYB proteins were interrupted by one or two introns, whereas 15 (6.1%) NtMYB genes did not hold any introns. Furthermore, more than eight introns were found in NtMYB112, AtMYB88, and AtMYB124, which were all clustered into S25.

In addition, the R2R3-MYB protein sequences of tobacco and *Arabidopsis* were submitted to the MEME tool to analyze the conserved motifs. As a result, a total of 10 motifs were identified, namely, motif 1–10 (Supplementary Figures S2, S3). Among them, motifs 2, 3, and 4 together constitute the R2R3-MYB domain, which could be found in all studied MYB members. Consistent with the results of gene structure analysis, the R2R3-MYB proteins in the same group usually have similar types and orders of motifs. In addition, several motifs were found to be unique to certain subgroups. For instance, motif 8 was only found in S31, while motif 9 was unique to S20, implying that these unique motifs might undertake different functions. The similarities in characteristic motifs in each group may reflect functional similarities and should be conducive to determining specific functions for each R2R3-MYB gene.

**Syntenic analysis of the tobacco R2R3-MYB gene**

Syntenic analysis is important in genome sequence comparison, which reveals the genomic evolution of different species, while the syntenic pairs are predicted as orthologs and might share similar functions (Wang et al., 2021). As a result, collinearity pairs of the R2R3-MYB member were found in tobacco and five other species (Figure 2A). The collinear pairings between 81 of the NtMYB genes with MYB members in *Arabidopsis* were identified, followed by 151 NtMYB genes pairing with tomato, 125 NtMYB genes with potato, and 23 and 10 NtMYB genes with MYB genes from rice and maize, respectively. Notably, more R2R3-MYB collinearity pairs were found between tobacco and dicotyledonous species than monocotyledonous species. Furthermore, a total of six tobacco MYB genes were identified to form collinear pairs with MYB genes from all of the other species, indicating that these MYB genes may have existed before the divergence of these species (Figure 2B). Interestingly, 29 R2R3-MYB collinear pairs were predicted between tobacco and three dicotyledonous plants, but not found in the tested monocotyledonous plants, suggesting that these 29 pairs might arise after the divergence of dicotyledonous
and monocotyledonous plants. The details of the tobacco MYB syntenic pairs can be found in Supplementary Table S5.

Chromosomal distribution and duplication events

The chromosomal location information of 246 NtMYB genes was obtained from the SGN database and visualized using the R (Figure 3A). As a result, Chromosome 04 harbored the most R2R3-MYB genes, while Chromosomes 01, 07, 11, 16, and 18 were found to hold only three R2R3-MYB genes. According to the previous definition of tandem gene events (Li et al., 2018), a total of seven clusters (NtMYB022/023, NtMYB023/024, NtMYB089/090, NtMYB118/119, NtMYB155/156, NtMYB171/172, NtMYB188/189) were identified, the first four of which were located on chromosomes and the others were found on scaffolds. Furthermore, NtMYB022/023 and NtMYB171/172 were found to arise from the tandem duplication events (Figure 3A).

The gene segmental duplication event served as the important access for plants to acquire new genes and gene family expansion. As results, a total of 64 tobacco R2R3-MYB genes were identified to form 42 segmental duplication pairs (Figure 3B and Supplementary Table S6). Notably, these results suggested that about 44% of the NtMYB genes may be generated by duplication events, which played the major role in the expansion of the MYB gene family in tobacco.

Promoter element analysis

In a previous study, the MYB genes had been reported to be involved in various developmental and stress responses. Considering these clues, the cis-element analysis was investigated to explore the probability of these NtMYB genes in developmental and stress responses. To study the expression regulation of the NtMYB genes, the promoter region of 246 R2R3-MYB genes was analyzed using the PlantCARE Online toolbox. Generally, it was found that a lot of cis-elements involved in various developmental and stress responses were present in these tobacco R2R3-MYB gene promoters. Furthermore, 13 cis-elements were selected from the PlantCARE database for visualization (Supplementary Figure S4). As a result, a total of 203 (82.5%) NtMYB gene promoters contained A B R E (abscisic acid responsive cis-element), suggesting that those R2R3-MYB genes may function in the abscisic acid signal pathway. Besides, 203 (82.5%) NtMYB gene promoters harbored E R E (ethylene responsive cis-element), while 129 (52.4%) gene promoters possessed a salicylic acid responsive cis-element (TCA-element). In addition, both C G T C A-motif and T G A C G-motif are related to MeJA responsive, and 184 and 185 gene promoters were detected to possess these two kinds of cis-element, respectively. Further, a total of 98 (39.8%) gene promoters were detected to hold the C A T-box, which was related to the development of the plant meristem. Notably, the MYB family members were reported to be induced by stress treatments; the stress response-related cis-elements of tobacco MYB gene promoters were also analyzed.
FIGURE 2
The synteny analysis of R2R3-MYB genes between tobacco and five representative species. (A) The gray line in the background represents the collinear blocks between tobacco and five representative species, while the collinear R2R3-MYB gene pairs are highlighted in red color. (B) The R2R3-MYB genes form the syntenic pairs between tobacco and all the other selected species, which is visualized by the Venn plot.
consequently. The stress-responsive cis-elements including MBS (MYB-binding site), TC-rich repeats, HSE (heat stress-responsive element), LTR (low-temperature-responsive element), WUN-motif (wound-responsive element), and ARE (anaerobic induction element) were found to be abundant in the promoter regions of many NtMYB gene promoters.

Interestingly, a total of 151 NtMYB gene promoters were predicted to hold W-box cis-elements, which act as the binding sites of the WRKY transcription factor, implying these NtMYB genes might be regulated by a certain WRKY transcription factor. Overall, the promoters of NtMYB genes possess abundant cis-elements, suggesting that the expression of these NtMYB genes might be regulated by multiple factors.

Expression analysis of the NtMYB genes from RNA-seq

To explore the expression pattern of the newly identified NtMYB genes, their RNA-seq data (GSE95717) was analyzed and visualized by R packages. As a result, the expression levels of 246 NtMYB genes in root, shoot, and shoot apex tissues were investigated (Figure 4). The results showed that several genes were expressed abundantly in the tested tissues, including NtMYB181, NtMYB216, and NtMYB067; while NtMYB176, NtMYB048, NtMYB026, NtMYB151, NtMYB227, NtMYB054, and NtMYB098 were detected to be highly expressed in the shoot and root. In addition, a total of 51 genes such as NtMYB033, NtMYB088, and NtMYB187 were only detected in the root, whereas 15 genes like NtMYB168, NtMYB154, and NtMYB096 were found to be expressed in all tested tissues except the root. It was worth noting that 61 (24.8%) NtMYB genes were not detected in these tested tissues, implying those genes might function in other tissues.

In addition, another RNA-seq data (SRP102153) was also used to analyze the expression pattern of NtMYB genes in different developmental stages of tobacco leaves. As a result, a total of eight leaf growth stages were sequenced, and the expression of 57 genes increased as tobacco leaves grew (Figure 5). In particular, NtMYB008 and NtMYB178 was only expressed in the later stages of leaf growth, suggesting they might have a certain relationship with leaf senescence. AtMYB2 was reported to contribute to the regulation of whole plant senescence (Guo and Gan, 2011), so its homologous genes were also concerned during the senescence of tobacco leaves. Combined with evolutionary analysis, it was found that genes homologous to AtMYB2 were expressed at a high level during leaf senescence, such as the NtMYB060, NtMYB079, NtMYB127, NtMYB084, and NtMYB177. This finding proved the reliability of evolutionary analysis on the one hand and implied the potential function of these genes in regulating leaf senescence.

The validation of expression patterns by qRT-PCR

To enrich the expression profile of the NtMYB genes of RNA-seq data, qRT-PCR was hired to analyze the expression changes of several representative genes. As a result, NtMYB096, NtMYB109, and NtMYB124 were observed to highly be expressed in the root, NtMYB053 showed abundant transcripts in the stem, and NtMYB130 was found to be expressed globally
FIGURE 4
Expression profiles of the NMYB genes in three different tissues (root, shoot, and shoot apex). The heatmap was constructed based on the transcriptome data of NMYB genes and visualized by R.

FIGURE 5
Expression profiles of the NMYB genes of middle tobacco leaves in different periods. The example of middle leaf harvested from plants from 15 to 85 DAT (days after topping). The heatmap was constructed based on normalized RNA-seq data of NMYB genes and visualized by R. Red indicates high expression, and blue indicates no detected expression. H indicates Honghuadajingyuan; M, middle leaves. * indicates the genes mentioned in the text.
in all tested tissues (Figure 6A), which were consistent with the RNA-seq data. In addition, NtMYB060 and NtMYB079 were detected to be upregulated during leaf senescence; on the contrary, NtMYB210 had down-regulated expression, implying those genes might participate in leaf senescence of tobacco. Notably, qRT-PCR added the floral tissue to enrich the expression profile of tobacco R2R3-MYB genes; the results showed that the selected genes were expressed in varying degrees in flowers. Especially, NtMYB105, NtMYB108, and NtMYB149 were highly expressed in flowers, suggesting they might play the key role in the flower development of tobacco.

Furthermore, a number of R2R3-MYB transcription factors were reported to respond to abiotic stresses in Arabidopsis; representative NtMYB genes were selected to test whether they could respond to abiotic stresses, including salt and drought stress. As a result (Figures 6B, C), NtMYB078 was significantly induced by salt and drought treatments, whereas NtMYB168 and NtMYB177 were repressed by salt treatment. The expression of several NtMYB genes continued to increase under drought treatment, such as NtMYB123, NtMYB201, and NtMYB210. Interestingly, the expression of NtMYB081 in response to salt stress was time-specific, reached the peak at 1 h of salt treatment, and then dropped sharply. Notably, NtMYB102 was found to respond significantly to both drought and salt treatments, whereas NtMYB010 was induced by drought treatment but nearly kept the original expression under salt stress.

**Subcellular localization analysis**

To explore the potential function of the NtMYB genes, the subcellular localization of the one of the salt-responsive genes, NtMYB102, was analyzed (Supplementary Figure S5). The full-length coding sequence of NtMYB102 without the stop codon was fused to the GFP reporter gene sequence, which was driven by the CaMV35S promoter. The Agrobacterium cultures with the NtMYB102-GFP fusion construct and the 35S::GFP control were transiently expressed in the leaves of N. benthamiana, respectively. As shown by confocal microscopy, the signal of GFP protein was found to distribute throughout the whole cell, whereas the fluorescence signal of the NtMYB102-GFP fusion protein was specifically confined within the nucleus, which was confirmed by staining with DAPI.

The function of NtMYB102 in plant drought and salt tolerance

As the transcriptional activation assay result, all the yeast cells grew well on a SD/-Trp medium, while on the SD/-Trp medium supplemented with X-α-Gal, the yeast cells harboring the NtMYB102 grew well and displayed a blue color; the yeast cells containing the pBridge empty vector were not in blue color (Figure 7A). These results showed that NtMYB102 has transactivation activities. Furthermore, the function of NtMYB102 gene was further examined through genetic experiments. Considering that this gene could be induced by drought and salt stresses, the overexpression lines and wild-type seedlings were firstly treated by drought stress. After the drought treatment, overexpression lines and wild-type seedlings displayed leaf wilting phenotypes, whereas the wild-type seedlings were much more extreme than those overexpression lines (Figure 7B and Supplementary Figure S6) and the survival rates of the OE-1 lines, OE-4 lines, and OE-5 lines were significantly higher than those of the wild-type seedlings (Figure 7C). Besides, the overexpression lines displayed higher water content than wild-type during dehydration (Figure 7D).

Furthermore, the salt tolerance of wild-type and NtMYB102 overexpressing tobacco was examined via root elongation assay (Figure 8). As a result, no significant difference in root length between wild-type and the NtMYB102 overexpressing plants was found under normal conditions. However, on 100 mM NaCl plates, longer roots of the independent overexpression lines were observed compared to the wild type. Hence, the overexpression of NtMYB102 gene could improve the drought and salt tolerance in transgenic tobacco.

**Discussion**

The MYB transcription factors play important roles in plant development, metabolism, and responding to biotic and abiotic stress (Stracke et al., 2007; Dubos et al., 2010). In this study, the newly identified R2R3-MYB members were studied through a series of analyses. In addition, the MYB genes homologous between Arabidopsis and tobacco were studied to investigate their potential functions.

A total of 246 R2R3-MYB members were identified from tobacco, and divided into 33 subgroups together with Arabidopsis R2R3-MYB members (Figure 1). The syntenic analysis could visualize the location of the homologous or orthologous genes and the presence of collinear R2R3-MYB genes in different species may have conserved functions, which gives an insight into the functions of the R2R3-MYB genes (Li et al., 2019). In the current study, we identified the collinear pairs of the R2R3-MYB genes in five studied species. A total of six tobacco R2R3-MYB genes were identified to form collinear pairs with genes from all the other species (Figure 2), whereas those six collinear R2R3-MYB genes were distributed in different subgroups (Figure 1), suggesting these R2R3-MYB genes may have existed before the divergence of these species. Gene duplication has played a very important role in the expansion of gene families (Kent et al., 2003; Cannon et al., 2004). In the current study, a total of 44 duplication events were identified in the 68 NtMYB genes, most (42) of which involved segmental duplication, and several (2) of which involved tandem...
FIGURE 6
The qRT-PCR analysis of representative NtMYB genes. (A) To verify the tissue specificity expression of the representative NtMYB genes, the expression level of each NtMYB gene was calculated relative to the root. (B) The expression level of representative NtMYB genes under salt stress treatments. (C) The expression level of representative NtMYB genes under drought stress treatments.
FIGURE 7
The function of NtMYB102 in drought tolerance. (A) Transactivation analysis of NtMYB102 in yeast. (B) Phenotypes of overexpression lines and wild-type tobacco under drought stress. The overexpression lines displayed higher drought tolerance after 2 weeks of drought stress and three days of recovery. (C) The survival rates of the overexpression lines and wild-type tobacco. (D) The water content of detached leaves of overexpression lines and wild-type tobacco, which was calculated by comparing the weight of the leaves before/after the treatments. The values represent means ± SD. *p < 0.05, **p < 0.01 (t-tests).
duplication. This discovery implied that duplication events might play an important role in the evolution of the tobacco R2R3-MYB gene family.

The R2R3-MYB transcription factors have been reported to be related to plant development (Stracke et al., 2001; Pucker et al., 2020). In subgroup 14, AtMYB37, which functions in axillary meristem development (Keller et al., 2006), clustered together with NtMYB053, and their coding genes were investigated to form the collinear pair (Figures 1, 2). Interestingly, NtMYB053 was highly expressed in the stem (Figure 1), suggesting that it might be involved in tobacco stem development, while NtMYB124 clustered together with AtMYB68 (Figure 1), which specifically regulates root growth (Feng et al., 2004), and the expression profiling showed NtMYB124 have high expression at the root (Figure 4), suggesting that it might be involved in root development.

In subgroup 18, AtMYB33 and AtMYB65 redundantly facilitate anther development (Millar and Gubler, 2005); NtMYB105, NtMYB222, NtMYB210, NtMYB149, NtMYB108, and NtMYB130 were clustered together with AtMYB33 and AtMYB65, and their coding genes were highly expressed in flowers (Figures 1, 4), suggesting they may be involved in the development of floral organs. These results suggested functional conservation between homologous R2R3-MYB members from Arabidopsis and tobacco.

Besides, several MYB members have been reported to control the synthesis of the anthocyanins, proanthocyanidins, flavonols, and flavonoids in plants (Mehrtens et al., 2005; Cao et al., 2021). Notably, AtMYB11/PFG2, AtMYB12/PFG1, and AtMYB111/PFG3 from subgroup 7 were characterized as specific flavonol regulators in Arabidopsis; AtMYB12 controls flavonol biosynthesis mainly in the root, while AtMYB111, primarily in cotyledons (Stracke et al., 2007). Evolutionary analysis showed that NtMYB096 and NtMYB109 were clustered together with these PFG members (Figure 1). Meanwhile, their coding genes were predicted to form five collinear gene pairs with AtMYB11, AtMYB12, and AtMYB111 (Figure 2). Further, the transcriptome and qRT-PCR data showed that NtMYB060 and NtMYB079 had been predicted to form a collinear gene pair with AtMYB2 and AtMYB108 (Figure 1). The transcriptome data showed that these tobacco homologous genes were detected to be up-regulated during tobacco leaf senescence (Figure 5), suggesting that they might also participate in the regulation of leaf senescence of tobacco. Interestingly, although NtMYB070 and NtMYB084 were predicted to arise from segmental duplication events (Figure 3B), NtMYB070 had been reported to be involved during tobacco leaf senescence (Figure 5), indicating these two duplicated genes might undergo subfunctionalization.

Many R2R3-MYB family members were found to confer tolerance to abiotic and biotic stresses in plants. In subgroup 1, NtMYB103 was clustered with AtMYB30, and they were detected to form a collinear gene pair (Figures 1, 2). AtMYB30 had been reported to be involved in abiotic stress responses (Marino et al., 2013). Interestingly, its tobacco homologous gene, NtMYB103, was detected to be induced by salt treatment (Supplementary Figure S4), implying that NtMYB103 might be involved in abiotic stress responses of tobacco. In subgroup 2, overexpression of AtMYB15 improves drought and salt tolerance in Arabidopsis (Agarwal et al., 2006). The collinearity analysis showed that NtMYB078, NtMYB082, NtMYB117, and NtMYB123 in the same subgroup were investigated to be forming the collinear gene pair with AtMYB15 respectively (Figure 3B). Similarly, those NtMYB genes were induced by multiple abiotic stress treatments (Figures 6B, C), implying that the R2R3-MYB members in this subgroup might also confer stress tolerance in tobacco.

In subgroup 22, NtMYB102 clustered together with AtMYB44, AtMYB70, AtMYB73, and AtMYB77 (Figure 1), those Arabidopsis members were reported to function in regulating stomatal closure and abiotic stress responses (Jung et al., 2008). Interestingly, NtMYB102 were found to form the collinear gene pairs with those Arabidopsis members (Figure 2) and the promoter analyses revealed that the NtMYB102 promoter region contains many ABRE cis-elements (Supplementary Figure 4), suggesting that it might be involved in ABA signalling and stress response. Notably, NtMYB102 had high transactivation activities in yeast and the NtMYB102-GFP fusion protein was in the nucleus (Figure 7A and Supplementary Figure S5). Furthermore, NtMYB102 was significantly induced by drought and salt stresses. In addition, the overexpression analyses further demonstrated that NtMYB102 can confer drought and salt tolerances in transgenic tobacco plant (Figures 7, 8). Those clues indicated that NtMYB102 acts as a transcriptional activator to regulate gene expression in response to stresses.
Conclusions

The systematic analysis of the tobacco genome sequences in this study was carried out to identify and characterize the R2R3-MYB genes; the phylogeny and expression profiling analysis implied that the tobacco R2R3-MYB gene family might be involved in various biological processes. The R2R3-MYB members homologous between Arabidopsis and tobacco were found to play conserved roles in regulating plant development and stress responses. Notably, NtMYB102 was found to be a nucleus-localized transcription factor with transactivation, and the coding gene was induced by salt treatments. Furthermore, the overexpression of NtMYB102 in tobacco significantly enhanced the drought and salt stress tolerance of the transgenic plants.

Data availability statement

Datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.
Author contributions

XL, CG, and ZL conducted the research and drafted the manuscript. The other authors assisted in data collection and analysis. WP and LW conceived the research and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

XL, GW, LC, JG and WP were employed by China Tobacco Hunan Industrial Co., Ltd. CG was employed by Yunnan Provincial Tobacco Company. GW was employed by Yuxizhongyan Tobacco Seed Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.998606/full#supplementary-material

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