Zhou G *et al.* (2023)
Notulae Scientia Biologicae
Volume 15, Issue 4, Article number 11649
DOI:10.15835/nsb15411649
Research Article

Identification and transcriptome analysis of the R2R3-MYB gene family in *Haloxylon ammodendron*

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Abstract

The MYB transcription factor family is widespread in plants and plays an important role in plant growth and development as well as in plant responses to stress. The MYB transcription factor family has been identified in a variety of organisms; however, it has not been identified and analysed in the desert plant *Haloxylon ammodendron*. In this study, R2R3-MYB genes were identified and analysed using a bioinformatic approach. A total of 78 R2R3-MYB genes were identified and named according to their position on the chromosome. The R2R3-MYB genes were unevenly distributed on nine chromosomes. Phylogenetic analysis showed that the HaMYB genes were all divided into 31 subfamilies. Covariance analysis revealed the presence of three pairs of fragmentary duplicated genes in *H. ammodendron* (*HaMYB54* and *HaMYB17*, *HaMYB44* and *HaMYB36*, *HaMYB42* and *HaMYB27*). Gene structure and conserved structural domain analysis revealed different subgroups with different orders of magnitude of variation in gene structures and conserved structural domains. Analysis of *cis*-elements showed that the *cis*-acting elements of HaMYBs were mainly associated with hormone and abiotic stress responses. Real-time quantitative PCR was used to detect the expression levels of HaR2R3-MYB genes, and six HaR2R3-MYB genes were found to respond to salt stress and six HaR2R3-MYB genes to drought stress, with *HaMYB22* and *HaMYB27* showing upregulated expression under both stresses. Transcriptome analysis showed that *HaMYB63* was significantly differentially expressed in the assimilated branches of *H. ammodendron*, and the subcellular localization of this protein showed that it was located in the nucleus and had transcriptional self-activating activity. These results provide a theoretical basis for further studies on the functions of the R2R3-MYB gene family and the molecular mechanisms of resistance in *H. ammodendron*.

Keywords: *Haloxylon ammodendron*; R2R3-MYB family; plant stress resistance; RNA-seq; bioinformation

Received: 07 Aug 2023. Received in revised form: 10 Oct 2023. Accepted: 01 Nov 2023. Published online: 15 Nov 2023.
From Volume 13, Issue 1, 2021, Notulae Scientia Biologicae journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Introduction

Haloxylon ammodendron is a shrub or small tree of the genus Haloxylon and a typical C4 plant whose leaves have degenerated into assimilated branches, which exercise photosynthesis instead (He *et al.*, 2018; Lü *et al.*, 2019). This species is distributed mainly in the deserts of Central Asia and Xinjiang, China (Li *et al.*, 2019), with many characteristics, such as drought, high temperature, salinity, wind erosion and cold resistance (Zhang *et al.*, 2016; Gao *et al.*, 2020; Lü *et al*., 2022). It plays an important role in maintaining the structure and function of desert ecosystems, such as blocking wind, promoting sand fixation, and improving the climate (Ma *et al.*, 2021), and is known as the "guardian of the desert". It also has some economic value as a host for the precious Chinese herb Cistanches, and it can be used for firewood and livestock fodder in Xinjiang and other places (Song *et al.*, 2006).

Transcription factors (TFs), also known as trans-acting factors, play a crucial role in the regulation of plant growth and development, acting as regulatory switches for genes that are involved in the regulation of gene expression in response to various environmental factors, thus ultimately controlling the metabolic processes of the plant body (Manna *et al.*, 2021; Romani and Moreno, 2021). The first MYB gene identified was *V-myb*, an oncogene derived from avian myeloblastoma virus (Klempnauer *et al.*, 1982), and the first MYB gene identified in plants was *ZmMYBC1* in maize (Paz-Ares *et al.*, 1987). The MYB transcription factor family contains a conserved structural domain, the MYB DNA-binding domain, which is divided into two distinct regions, the N-terminus and the C-terminus, with the N-terminus being the conserved MYB DNA-binding domain and the C-terminus being the transcriptional regulatory domain, which is not conserved. The MYB DNA-binding domain usually consists of 1-4 incompletely repeated amino acid repeats (R), each typically consisting of 53 amino acids, which form three α-helices. The second and third helices are folded helix-turned-helical (HTH) structures, forming a hydrophobic structure through which MYB transcription factors bind to the major groove of the target gene and thus play a regulatory role (Stracke *et al.*, 2001). Depending on the number of MYB protein repeats (R), they are generally classified into four categories, including MYB proteins containing single or partial MYB repeats (these MYB genes are collectively referred to as "MYB-related"), R2R3-MYBs containing two repeats (R2R3), R1R2R3s containing three repeats, and MYB-related proteins that mainly play regulatory roles in cellular morphological changes, secondary metabolism, and chloroplast development (Ambawat *et al.*, 2013); the upstream promoter region of 3R-MYB is rich in mitosis-specific activator core sequences, which mainly play an important role in plant cell cycle control. They also participate in plant abiotic stress responses (Feng *et al.*, 2017). Among them, 4R-MYB transcription factors are a less studied class in plants; R2R3-MYB transcription factors are the most widely distributed and abundant among plant species and therefore the most studied class of MYB genes (Sun *et al.*, 2019; Dubos *et al.*, 2010). R2R3-MYB genes have been identified in a variety of organisms, and in addition to 126 R2R3-MYB genes identified in the model plant *Arabidopsis*, 110, 157, 108, 207, and 70 R2R3-MYB genes have been identified in rice, maize, grape, poplar, and sugar beet, respectively (Matus *et al.*, 2008; Du *et al.*, 2012; Katiyar *et al.*, 2012; Stracke *et al.*, 2014; Zhao *et al.*, 2020). However, there are no reports on the identification of the R2R3-MYB gene family in *H. ammodendron*.

R2R3-MYB transcription factors are involved in a variety of biological processes in plants. including plant physiological and biochemical processes such as primary and secondary metabolic processes. Yang *et al.* (2022) found that the R2R3-MYB-like transcription factor VcMYB4a in blueberry regulates lignin biosynthesis, and Zhu *et al.* (2020) found that *CmMYB8* in chrysanthemum negatively regulates lignin biosynthesis and flavonoid biosynthesis. R2R3-MYB transcription factors are also involved in phytohormone response processes (Stracke *et al.*, 2001). The R2R3-MYB transcription factor in buckwheat is induced by abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) hormones (Gao *et al.*, 2016). RhMYB108 in rose acts as an effector of ethylene and JA to regulate petal senescence (Zhang *et al.*, 2019), and the *Arabidopsis*

transcription factor MYB77 regulates growth hormone signalling (Shin *et al.*, 2007). R2R3-MYB transcription factors are also involved in plant responses to biotic and abiotic stresses (Stracke *et al.*, 2001). Shriti *et al.* (2023) found that overexpression of the chickpea CaMYB78 transcription factor enhanced resistance to *Fusarium acanthoides*, and overexpression of the wild soybean R2R3-MYB transcription factor *GsMYB15* in *Arabidopsis* enhanced resistance to salt stress and cotton bollworm (Shen *et al.*, 2018). The desert moss *Syntrichia caninervis* was found to have a large number of MYB genes differentially expressed in response to cold and frost damage, suggesting that these genes play an important role in the regulation of cold and frost stress responses in desert plants (Salih *et al.*, 2023). MYB transcription factors play an important role in dune reed adaptation to desert environments and an important role in resisting abiotic stress (Cui *et al.*, 2023). *CgMYB1* expression is induced by salt stress and cold stress in *Chenopodium glaucum*, which is a member of the family Chenopodiaceae, and overexpression of this gene in *Arabidopsis* increases salt tolerance and cold tolerance (Zhou *et al.*, 2023). PeR2R3s in poplar (*Populus euphratica*) plays an important role in ABA-mediated drought stress response (Sun *et al.*, 2023), but MYB transcription factors have been less studied in the desert plant *H. ammodendron*.

Herein, we identified the R2R3-MYB gene family in *H. ammodendron* by bioinformatics, and transcriptomic data analysis yielded the differentially expressed gene HaMYB63 for subcellular localization and transcriptional self-activation analysis, which may be involved in the abiotic stress response in *H. ammodendron*.

Materials and Methods

Plant materials

On July 28, 2021, at 14:00, assimilated branches of wild *H. ammodendron* with good and consistent growth and different colours and hardness levels were selected from the southern edge of the Gurbangtungut Desert (84°31'-98°80'E, 44°11'-46°21'N) in the Junggar Basin, Xinjiang, China. The green and soft assimilated branches and the reddish-green and hard assimilated branches (abbreviated as “GG” and “RG”, respectively) were selected from the southern margin of the wild *H. ammodendron* distribution. The collected samples were quickly placed in a liquid nitrogen tank, brought to the laboratory, stored in a -80 °C freezer, and sent to Biomarker Technologies for transcriptome sequencing, with three biological replicates selected for each set of samples.

The mature seeds of *H. ammodendron* were preserved in the laboratory, and full-grained and uniformly sized *H. ammodendron* seeds were selected for planting. First, 75% ethanol was added to the centrifuge tube containing the *H. ammodendron* seeds, the supernatant was pipetted for 30 s and aspirated, distilled water was added and the seeds were washed again with 75% ethanol, 5% NaClO solution was added once, and then distilled water was used 5-6 times until the seeds were cleaned. *H. ammodendron* seeds were placed in sterile triangular flasks containing 1/2 MS solid medium and incubated until the assimilated branches of *H. ammodendron* seedlings reached approximately 10 cm for drought and salt treatment. The assimilated branches were treated with 20% PEG6000 solution and 200 mmol/L NaCl solution at 25 °C for 0 h, 2 h, 4 h, 6 h, 12 h and 24 h. Three biological replicates were performed for each of the above experimental samples.

Identification of R2R3-MYB family members in the H. ammodendron genome

The genomic data and proteomic data of *H. ammodendron* were downloaded from Figshare (*https://doi.org/10.6084/m9.figshare.17128424.v1.*). Arabidopsis MYB protein sequences were downloaded from the Arabidopsis database TAIR (*https://www.arabidopsis.org/*). We obtained sequences from the Pfam database (*http://pfam.xfam.org/*) to download the Hidden Markov Model (HMM) of the MYB structural

domain (PF00249) and used HMMER software (*http://hmmer.org/*) (Potter *et al.*, 2018) to identify candidate MYB genes in H. ammodendron using an HMMER search. The obtained candidate genes were submitted to the NCBI-CDD database after removing redundant sequences (*https://www.ncbi.nlm.nih.gov/cdd/*) (Lu *et al.*, 2020), the Pfam database, and SMART (*http://smart.embl.de/*) for structural domain validation and further identification of MYB gene family members. The obtained MYB genes were named according to their position on the chromosome. The gene family was identified using the ExPASy online database ProtParam tool (*https://web.expasy.org/protparam/*) (Artimo *et al.*, 2012) to predict the number of amino acids, theoretical isoelectric point, lipid index and other relevant physicochemical properties by WoLF PSORT (*https://wolfpsort.hgc.jp/*) (Horton *et al.*, 2007) online software for protein subcellular localization prediction.

Chromosomal location of the H. ammodendron R2R3-MYB gene family

TBtools software (Version 1.09876) (Chen *et al.*, 2020) was used to extract chromosomal location information for members of the *H. ammodendron* MYB gene family, and Mapchart software (Version 2.32) (Voorrips, 2002) was used to map the chromosomal localization of the HaMYB gene.

Phylogenetic analysis of the H. ammodendron R2R3-MYB gene family

The MYB protein sequences of *Arabidopsis* and *H. ammodendron* were compared using multiple sequences in Clustal W of MEGA 6 (Tamura *et al.*, 2013) software, and then based on the results of the comparison, a phylogenetic tree was reconstructed using the ML method, where the bootstrap value was set to 2000. The resulting phylogenetic tree was visualized and beautified using the online tool Evolview (*https://evolgenius.info/*) (Subramanian *et al.*, 2019).

Covariance analysis of the H. ammodendron R2R3-MYB family

To determine the evolutionary history of the *H. ammodendron* R2R3-MYB family, the HaR2R3-MYB gene replication events were analysed using Multiple Collinear Scanning Toolkit (*MCScanX*) (Wang *et al.*, 2012), and the HaR2R3-MYB genes were covalently analysed with the R2R3-MYB genes in *Arabidopsis*, sugar beet, and spinach using TBtools software. The Dual Synteny Plot tool was applied to plot the covariance.

Conserved motifs and gene structure of the H. ammodendron R2R3-MYB gene family

Evolutionary trees were reconstructed for HaR2R3-MYBs using MEGA 6 software to obtain nwk files. The nwk files were obtained by MEME (Version 5.4.1) online software (*https://meme-suite.org/meme/doc/meme.html*) (Bailey *et al.*, 2009) for motif analysis (setting the number of conserved motifs to 10), with a minimum motif of 6 and a maximum motif of 50. All the above results were visualized using TBtools software.

Analysis of cis-acting elements in the promoter region of H. ammodendron R2R3-MYB genes

To better understand the function of the HaMYB gene, the 2000 bp DNA sequence upstream of the *H. ammodendron* MYB gene was extracted using TBtools software, using the PlantCARE database (*http://bioinformatics.psb.ugent.be/webtools/plantcare/html/*) in the Search for CARE tool to predict the possible *cis*-acting elements and TBtools software to visualize and analyse them.

qPCR

Based on the *H. ammodendron* cDNA sequence, the Primer-BLAST tool in the NCBI database (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome*) (Ye *et al.*, 2012) was used to design realtime fluorescence quantitative PCR (qPCR) primers (Supplementary Table 1). The expression of candidate genes was analysed by qPCR using *H. ammodendron* cDNA as a template, *18s rRNA*

was used as the internal reference gene, and three replicates were performed for each sample. Total RNA was extracted using the Biomarker Plant Total RNA Isolation Kit (Biomarker, China). Reverse transcription was performed using the BiomarkerScript II 1st Strand cDNA Synthesis Kit (Biomarker, China). qPCR experiments were performed using Biomarker 2X SYBR Green Fast qPCR Mix (Biomarker, China), and a Roche LightCycler96 Fluorescent PCR instrument was used for fluorescence quantification experiments. The conditions for fluorescence quantification experiments were 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Transcriptome data analysis

H. ammodendron assimilated branch RNA was extracted using the Total Plant RNA Extraction Kit (Biomarker, China), and the concentration and purity of RNA were examined to select high-concentration (concentration ≧ 300 μg/g) and high-purity ($1.8 < OD_{260/280} < 2.0$) RNA for subsequent sequencing analysis. Eukaryotic mRNA was enriched with magnetic beads with Oligo (dT) and randomly interrupted by adding fragmentation buffer. cDNA was obtained by reverse transcription using mRNA as a template, purified, end-repaired, A-tailed and ligated with sequencing junctions, and finally enriched by PCR to obtain cDNA libraries. After library construction, the effective concentration of the library (effective library concentration > 2 nM) was accurately quantified by qPCR to ensure the quality of the library, and finally, RNA sequencing was performed on *H. ammodendron* using the Illumina NovaSeq6000 high-throughput sequencing platform. The raw data obtained from the sequencing platform were filtered to obtain clean data, and the clean data were used for subsequent analysis. Using HISAT2 (Kim *et al.*, 2019) software, the clean data were compared with the reference genome to obtain mapped data, and the mapped data were used for subsequent analysis. Subsequently, the above Reads were assembled and quantified by StringTie software (Pertea *et al.*, 2015) comparison. After the comparative analysis, StringTie comparison was used to assemble and quantify the above Reads. DESeq2 (Love *et al.*, 2014) software with the Benjamini-Hochberg method was used to correct the p values (*p* values) obtained from the original hypothesis test during differential expression analysis. The p value was used as the key index for differentially expressed gene screening, and the conditions for screening differentially expressed genes were fold change > 1.5 and *p* value < 0.05. GO and KEGG annotation and enrichment analysis were performed on the screened differentially expressed genes. Transcriptome data validation qPCR primers are shown in Supplementary Table 1.

Gene isolation and plasmid construction

The CDS of *HaMYB63* was cloned using the high-fidelity enzyme DNA polymerase (Thermo Fisher, USA), the pCAMBIA1304 vector was selected as the subcellular localization vector, and *Nco* I and *Spe* I were selected as the digestion sites to construct the pCAMBIA1304-GFP-HaMYB63 subcellular localization vector GV3101. HaMYB63, the nuclear localization marker named HY5-RFP, and *Agrobacterium tumefaciens* OD_{600} (pSoup-19), which facilitates heterologous protein expression, were adjusted to 1.0 using MS suspension (containing 10 mM $MgCl_2$ and 100 μM acetosyringone) and subsequently mixed at a volume ratio of 1:1:1. Inject the mixture into the leaves of the tobacco, incubated for 24-48 h in the dark and observed at a wavelength of 488 nm using a laser confocal microscope (Zeiss, Germany).

The pGBKT7 vector was used as the self-activating vector. The *Nde* I and *Bam*H I endonucleases (Thermo Fisher, USA) were selected to linearize the pGBKT7 vector, and the self-activating vector was constructed using the homologous recombination kit (Vazyme, China) with the homologous recombination method. The vector plasmid was transferred into yeast AH109 receptor cells, and the yeast cells were cultured in SD-Trp-deficient medium, SD-Trp/-His/Ade-deficient medium and SD-Trp/-His/Ade-deficient medium containing X-α-gal at 28 °C for 2-3 d. Yeast growth and discolouration were observed to determine whether HaMYB63 has transcriptional self-activating activity.

Results

Identification and analysis of R2R3-MYB genes and chromosomal localization in H. ammodendron

To systematically study the copy number changes in the MYB gene family during the evolution of *H. ammodendron*, first, we used HMMsearch to comprehensively search MYB genes in the *H. ammodendron* genome, remove redundancies, screen a total of 151 sequences containing MYB conserved structures, screen the sequences containing only 2 repetitive sequences among the 151 sequences, and validate the screening results in the NCBI-CDD database and Pfam database and on the SMART website. Finally, we identified 78 R2R3-MYB genes in *H. ammodendron* and named them HaMYB01~HaMYB78 according to their position on the chromosome (Figure 1). Prediction of the physicochemical properties of *H. ammodendron* R2R3-MYBs revealed that their encoded proteins contain 172 (HaMYB06) to 1609 (HaMYB56) amino acid residues with relative molecular masses ranging from 19865.71 (HaMYB06) to 175360.27 (HaMYB56) and a theoretical isoelectric point of 4.82 (HaMYB42)~9.71 (HaMYB11), and subcellular localization showed that 73 R2R3-MYB proteins were localized in the nucleus, 2 in the cytoplasm and 3 in the chloroplast (Supplementary Table 2).

According to the *H. ammodendron* R2R3-MYB chromosome localization map (Figure 1), the HaR2R3-MYB genes were unevenly distributed on nine chromosomes, including chromosome 1, which contained the fewest R2R3-MYB genes at only 4, chromosome 7, which contained the most R2R3-MYB genes at 12, and 2 and 8, which contained 10 R2R3-MYB genes each. The number of R2R3-MYB genes on the remaining chromosomes varied, and most R2R3-MYB genes were distributed at both ends of the chromosomes.

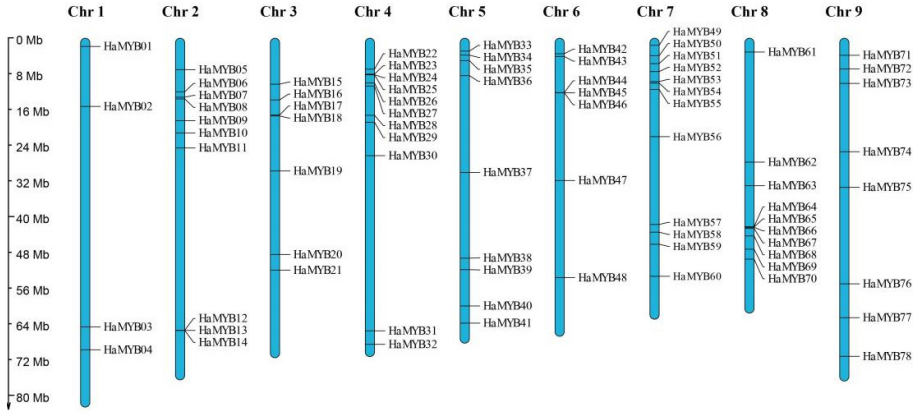

Figure 1. Chromosome localization map of the R2R3-MYB family of *H. ammodendron*

Evolutionary analysis of the R2R3-MYB genes of H. ammodendron

To further analyse the evolutionary relationships of the *H. ammodendron* R2R3-MYB genes, a phylogenetic tree was reconstructed between *Arabidopsis* R2R3-MYBs and *H. ammodendron* R2R3-MYBs (Figure 2). The phylogenetic tree was reconstructed using MEGA6 software for multiple sequence alignment with the ML method, in which the number of bootstrap replicates was set at 2,000 and Poisson correction was applied. The HaR2R3-MYB family was divided into 32 subgroups (named G1-G32) according to a classification scheme similar to that of *Arabidopsis* R2R3-MYBs. No HaR2R3-MYB members were found to be distributed in the two subgroups S15 and S12, while G1, G2, G3, and G4 contained only members from HaR2R3-MYB, and other HaR2R3-MYBs were mainly distributed in G8 (S21), G16 (S6+S5), G25 (S14), and G29 (S1). The number of *Arabidopsis* members in these subgroups was higher than that of *H. ammodendron* members. The fact that the number of members in these subgroups is higher than that of *H.*

ammodendron, sometimes up to twice as many, suggests that the R2R3-MYB members between *H. ammodendron* and *Arabidopsis* segregated during the evolutionary process.

Figure 2. Evolutionary analysis of the *H. ammodendron* R2R3-MYB family and *Arabidopsis* R2R3-MYB family. The triangles indicate *Arabidopsis* R2R3-MYB members, and the circles indicate *H. ammodendron* R2R3-MYB members.

Collinearity analysis of H. ammodendron R2R3-MYBs

To further investigate the evolution of the *H. ammodendron* R2R3-MYB family, we first performed a covariance analysis within the species (Figure 3 A). The analysis revealed the presence of gene duplication events in the *H. ammodendron* R2R3-MYB family, with three gene pairs undergoing fragmentary duplication events, including duplication between *HaMYB54* (*SS28368.t1*) and *HaMYB17* (*SS11433.t1*), *HaMYB44* (*SS24322.t1*) and *HaMYB36* (*SS19636.t1*), and *HaMYB42* (*SS23511.t1*) and *HaMYB27* (*SS15191.t1*), and no tandem duplication events were found. This indicates that some *H. ammodendron* R2R3-MYB genes are from gene duplication and that all of them are from fragment duplication.

We further performed covariance analysis of the *H. ammodendron* MYB genes with MYB genes of other species, including the model plant Arabidopsis and the plants sugar beet (*Beta vulgaris*) and spinach (*Spinacia oleracea*). The HaMYB gene is genetically homologous to those of other species, and the analysis revealed that the HaMYB gene is covariant with genes in Arabidopsis (55 direct homologous pairs distributed on all chromosomes), sugar beet (52 direct homologous pairs distributed on all chromosomes) and spinach (37 direct homologous pairs distributed on all chromosomes) (Figure 3 B).

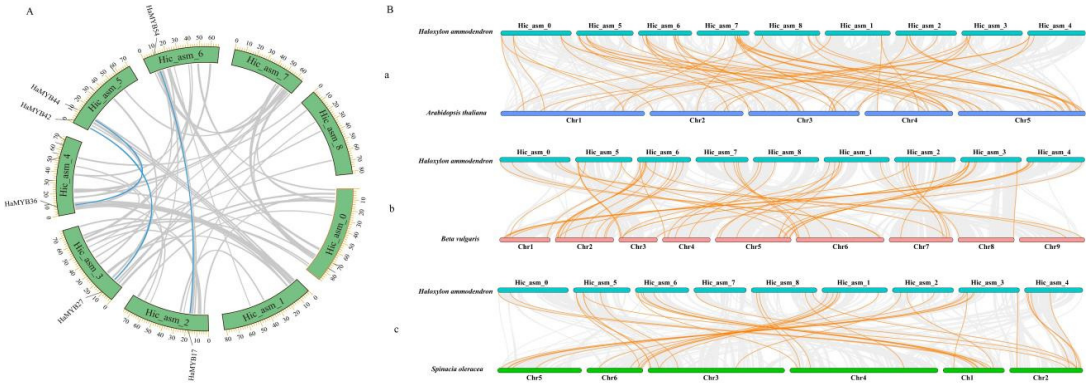

Figure 3. R2R3-MYB covariance analysis. (A) Analysis of *H. ammodendron* R2R3-MYB replication events. (B) Covariance analysis of *H. ammodendron* R2R3-MYBs with those of other species. (a) Covariance analysis of *H. ammodendron* with *Arabidopsis*; (b) covariance analysis of *H. ammodendron* with sugar beet; (c) covariance analysis of *H. ammodendron* with spinach.

Analysis of the structure and conserved sequences of H. ammodendron R2R3-MYB genes

To further analyse the composition of the *H. ammodendron* MYB genes, we mainly analysed their full-length protein sequences and conserved sequences (Figure 4, Supplementary Figure 1). Analysis of the conserved sequences of the *H. ammodendron* R2R3-MYB family revealed that most were at the 5' end. The number and distribution of conserved sequences of members in the same subgroup were relatively conserved, with five conserved sequences in subgroup G13, four conserved sequences in subgroup G1, three conserved sequences in subgroups G5-G12, and five to six conserved sequences in the remaining subgroups except for subgroups G2-G4, suggesting that the conserved sequences may be related to the exercise of MYB protein function. Structural analysis of the *H. ammodendron* R2R3-MYB genes revealed that they contain different numbers of introns, among which HaMYB01 has the highest number (11) of introns and the gene with the lowest number of introns has only one intron, for example, the other members of the G1 subgroup except for HaMYB66. This indicates the number of conserved sequences in *H. ammodendron* R2R3-MYBs is not directly related to the number of introns in the genes.

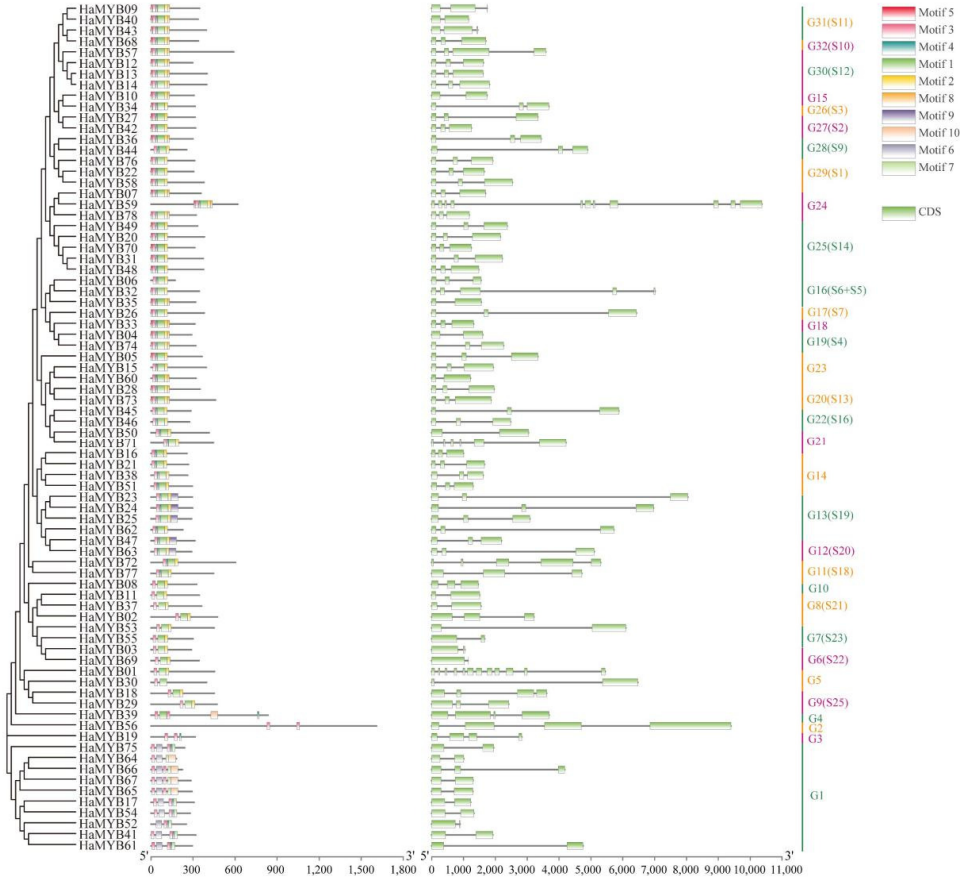

Figure 4. Conserved structure and gene structure analysis of *H. ammodendron* R2R3-MYB genes

Analysis of cis-acting elements of the H. ammodendron R2R3-MYB gene promoter

Transcription factors play a very important role in the regulation of gene expression, mainly by recognizing specific DNA sequences to regulate the expression of downstream genes at different spatial and temporal levels. Analysis of the *cis*-acting elements in the HaR2R3-MYB gene promoter showed that there are various numbers of *cis*-acting elements in the HaR2R3-MYB transcription factors, which are divided into two main categories, *cis*-acting elements related to plant hormones, such as SA, JA, auxin, ABA, and gibberellin (GA)-related *cis*-acting elements, and *cis*-acting elements associated with abiotic stresses, such as defence and stress response elements and low-temperature response elements. Hormone-related *cis*-acting elements were present in the promoter regions of almost all HaR2R3-MYB genes, with most hormone-response-related elements responding to JA and ABA and approximately the same numbers of both *cis*-acting elements associated with abiotic stresses (Figure 5). This indicates that the H. ammodendron R2R3-MYB genes are likely to be involved in different hormone signalling pathways, with most of them involved in JA and ABA signalling, as well as in the abiotic stress response in plants, with an important overall role in plant stress-related processes.

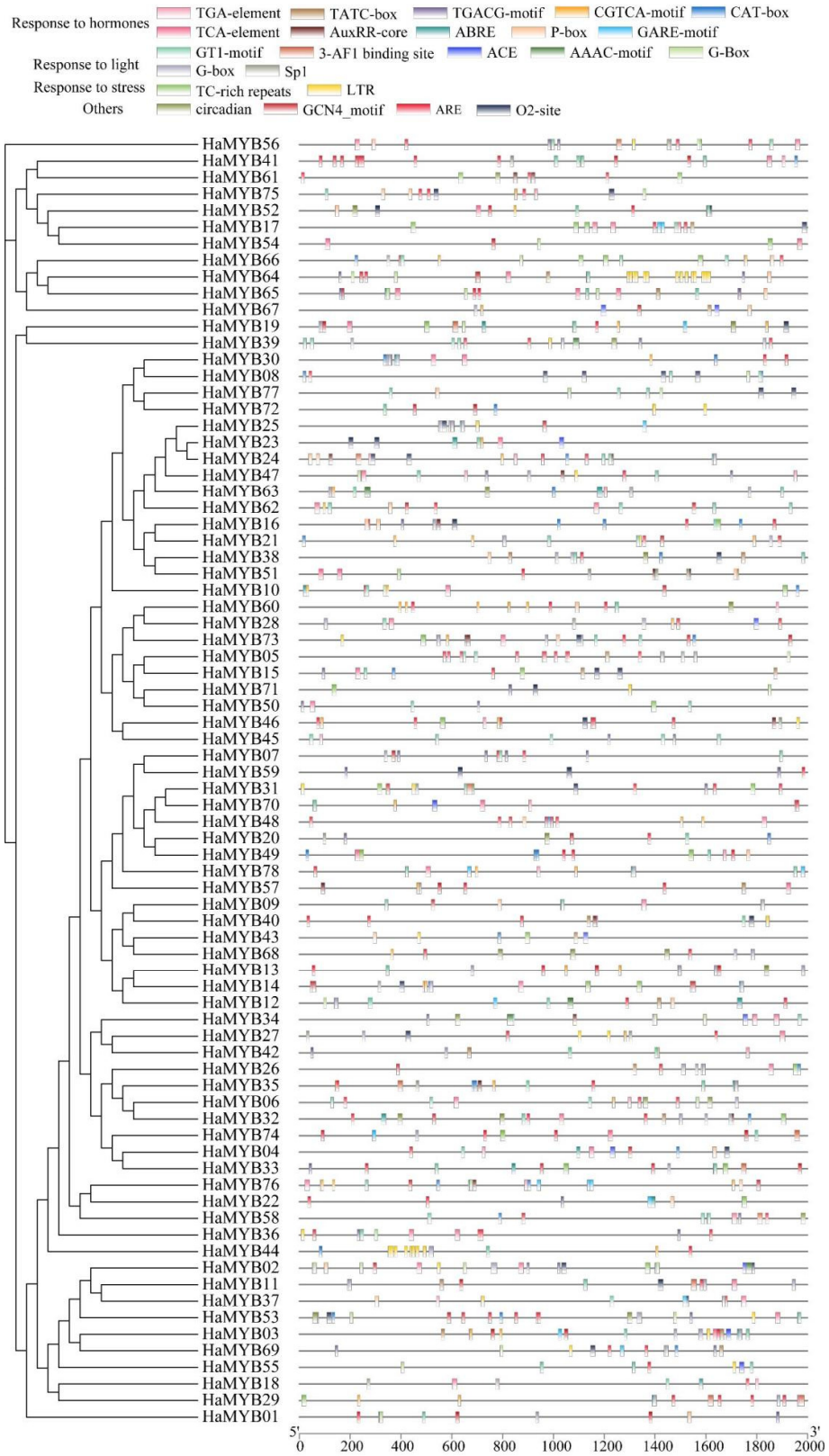

Figure 5. Analysis of *cis*-acting elements in *H. ammodendron* R2R3-MYB genes

qPCR of H. ammodendron R2R3-MYB genes under drought and salt stress

To investigate the role of HaR2R3-MYBs in *H. ammodendron* stress resistance, we used PEG6000 and NaCl solutions to simulate drought and salt stress, respectively. Based on the evolutionary analysis of R2R3-MYBs in Arabidopsis and *H. ammodendron* and reports related to the resistance role of R2R3-MYB genes in *Arabidopsis*, we finally chose to aggregate the subgroups G27 (S2), G29 (S1), and G30 (S11) (*HaMYB12*, *HaMYB13*, *HaMYB14*, *HaMYB22*, *HaMYB27*, *HaMYB42*) under salt stress. qPCR experiments were performed under salt stress conditions (Figure 6 A), and six genes clustered in subgroups G27 (S2), G29 (S1), and G8 (S21) were selected for qPCR analysis under drought conditions: *HaMYB02*, *HaMYB11*, *HaMYB22*, *HaMYB27*, *HaMYB37*, and *HaMYB42* (Figure 6 B, Supplementary Table 1). The qPCR results showed that the expression of these genes varied at different periods, and their expression was higher than that at 0 h at different periods of abiotic stress, suggesting that these genes are involved in the resistance response of *H. ammodendron*. Among them, *HaMYB22*, *HaMYB27*, and *HaMYB42* showed significant changes in expression under both drought stress and salt stress conditions, suggesting that *HaMYB22*, *HaMYB27*, and *HaMYB42* are involved in both the salt stress response and the drought stress response of *H. ammodendron*.

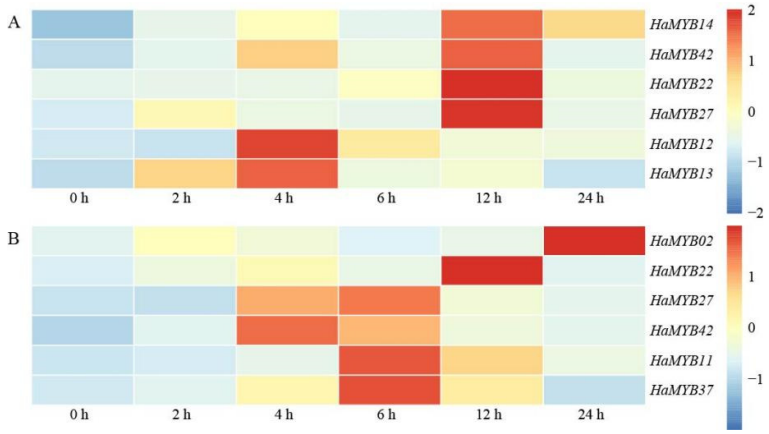

Figure 6. Expression analysis of *HaMYB* genes under stress. (A) Expression analysis of *HaMYB* genes under salt stress. (B) Expression analysis of *HaMYB* genes under drought stress

Transcriptome data analysis

The differentially expressed genes were screened with |log2-fold change|≥1.5 and p value < 0.05, and the results revealed 2505 differentially expressed genes in GG and RG, including 1569 upregulated genes and 936 downregulated genes (Figure 7 A). GO and KEGG annotation analysis and enrichment analysis were performed for all genes. GO enrichment of differentially expressed genes (Figure 7 B) showed that differentially expressed genes were mainly enriched in cellular process, metabolic process, single-organism process, and other biological processes. The cellular components mainly included cell, cell part, membrane and other components. The molecular function category mainly included binding, catalytic activity, transporter activity and other functions. The differentially expressed genes were found to be mainly enriched in the pathways of plant-pathogen interactions, phenylpropane metabolism, and starch and sucrose metabolism (Figure 7 C). The differentially expressed genes included MYB transcription factors, of which *HaMYB47* (*SS25656*), *HaMYB63* (*SS33758*), *HaMYB24* (*SS14971*), and *HaMYB28* (*SS15728*) showed significant changes in expression, with *HaMYB63* and *HaMYB28* showing higher expression in RG than in GG and *HaMYB47* and *HaMYB24* in GG than in RG. This suggests that these genes are involved in the secondary metabolic pathway of *H. ammodendron* in summer, causing the difference in colour and hardness of assimilated branches. To verify

whether the transcriptome data were reliable, we randomly selected 12 genes for qPCR experiments, and the results showed that the transcriptome data were reliable (Figure 7 D).

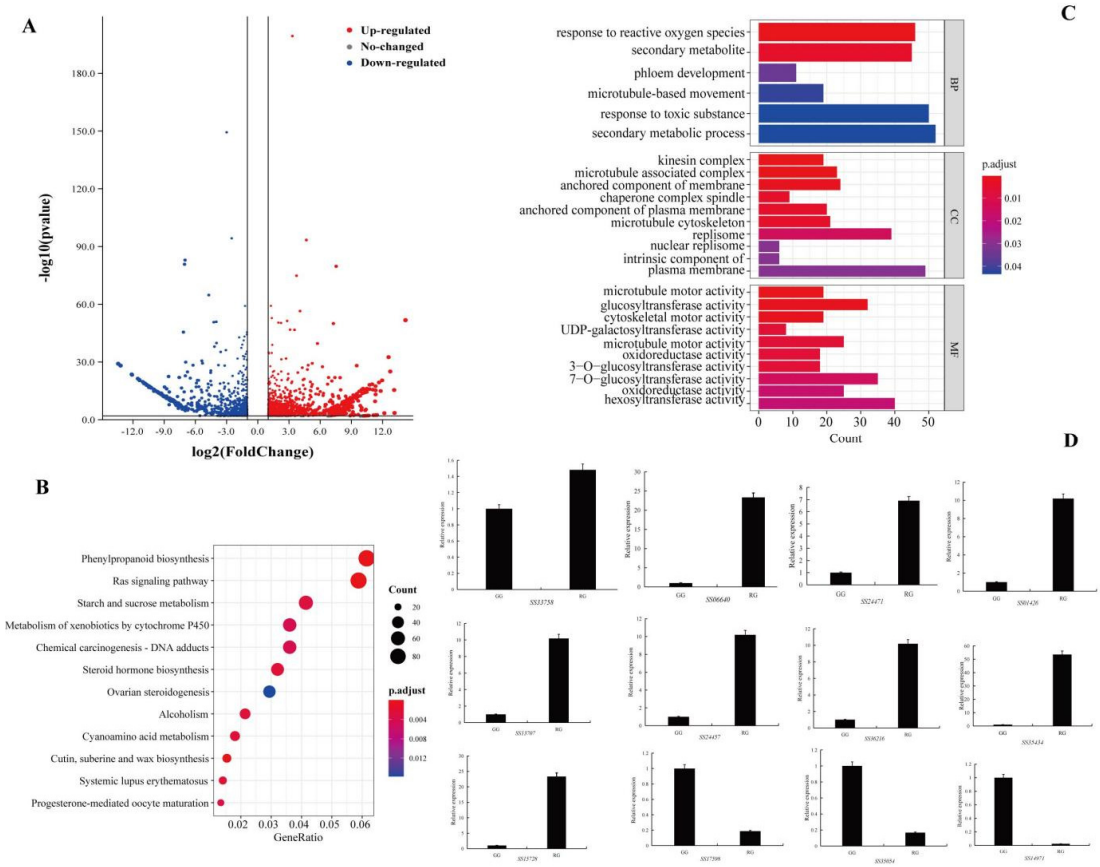

Figure 7. Transcriptome data analysis results. (A) Volcano plot of differentially expressed genes. (B) The results of GO enrichment analysis of differentially expressed genes. (C) The results of KEGG enrichment analysis of differentially expressed genes. (D) qPCR results of 12 differentially expressed genes.

Subcellular localization and transcriptional autoactivation analysis of HaMYB63

Based on the results of transcriptome data analysis, which showed that *HaMYB63* was significantly differentially expressed in GG and RG and showed upregulation in RG, it was hypothesized that this gene plays an important role in *H. ammodendron*, and therefore, the protein characteristics of *HaMYB63* were analysed.

To determine the subcellular localization of HaMYB63 protein in *H. ammodendron*, we obtained the CDS of *HaMYB63* by cloning using a high-fidelity enzyme (Thermo Fisher, USA), selected the pCAMBIA1304 vector as the subcellular localization vector, and selected *Nco* I and *Spe* I as the enzyme cleavage sites to construct the pCAMBIA1304-GFP-HaMYB63 subcellular localization vector to express HaMYB63 fused with GFP. Observations under laser confocal microscopy showed (Figure 8 A) that HaMYB63 was expressed in the nucleus, which was consistent with our WoLF PSORT (*https://wolfpsort.hgc.jp/*) online prediction results.

To analyse whether HaMYB63 has transcriptional self-activating activity, we constructed transcriptional self-activating vectors based on the CDS of *HaMYB63* and the pGBKT7 vector sequence, transferred the constructed vectors into the yeast AH109 receptor state, and grew yeast negative control and positive control and yeast containing HaMYB63 in SD-Trp-deficient medium to observe yeast growth (Figure

8 B). The fact that all three yeasts were able to grow indicates that all three vectors were transferred into yeast cells separately. The positive yeast and pGBKT7-HaMYB63 yeast could both grow on SD-Trp/-His/Ade-defective medium, the negative yeast could not grow normally, and the positive yeast and pGBKT7-HaMYB63 yeast grown on SD-Trp/-His/Ade-defective medium containing X-α-gal/Ade-deficient medium turned blue, while the negative yeast could not grow. These results suggest that the HaMYB63 protein has transcriptional self-activating activity.

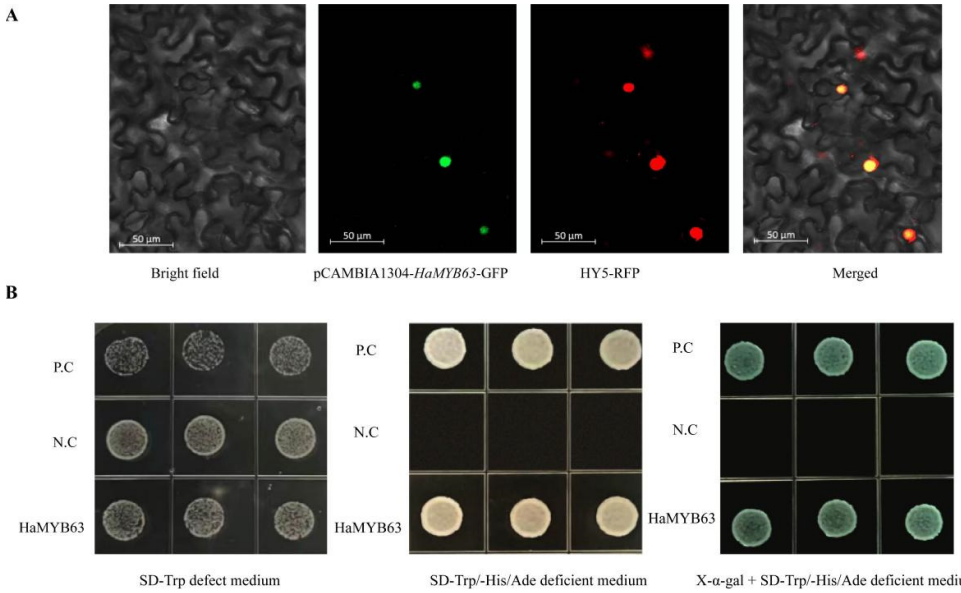

Figure 8. HaMYB63 protein characterization. (A) Subcellular localization results. (B) Transcriptional self-activation results. P.C. indicates positive control, N.C. indicates negative control.

Discussion

The *cis*-acting elements of promoters play a key role in activating gene expression, and analysis of the *cis*-acting elements of the HaMYB family revealed the highest number of *cis*-acting elements associated with the hormones JA and ABA and approximately the same number associated with defence and low-temperature stress. *MYB33*, *MYB65*, and *MYB101* are overexpressed in *Arabidopsis* and potato, leading to stomata showing hypersensitivity to ABA and thus improving tolerance to drought (Wyrzykowska *et al.*, 2022), and cotton R2R3-type MYB transcription factors positively regulate cotton resistance to yellow wilt through the lignin biosynthesis and JA signalling pathways (Zhu *et al.*, 2022). This suggests that HaR2R3-MYB family members are likely to regulate resistance to abiotic stresses in *H. ammodendron* by participating in the ABA and JA signalling pathways, while HaR2R3-MYB family members may also be involved in plant low-temperature stress and defence mechanisms.

R2R3-MYB genes have been shown to play an important role in response to biotic and abiotic stresses (Stracke *et al.*, 2001). At present, stress-related MYB genes have been identified in desert plants, such as *C. glaucum* and *P. euphratica* (Zhou *et al.*, 2023; Sun *et al.*, 2023). Under drought stress and salt stress, the expression of 6 genes in *H. ammodendron* was analysed by qPCR, and the expression levels of these genes were significantly increased under the two treatments, indicating that these genes may be involved in the abiotic stress response of *H. ammodendron*. In general, proteins clustered in the same subgroup have similar biological functions, and members of the S1, S2, S11, and S21 subgroups in *Arabidopsis*, such as *AtMYB60* and *AtMYB96*,

have been reported to be involved in the ABA signalling pathway and the physiological regulation of response to drought treatment (Rusconi *et al.*, 2013; Yao *et al.*, 2021), so the HaR2R3 homologues with S1, S2, S11, and S21 MYB genes may have similar functions. As shown in the present study, HaR2R3-MYBs in the S1, S2, S11, and S21 subgroups may be involved in salt stress and drought stress responses, and the functions of these genes were further verified by qPCR experiments.

Phylogenetic evolutionary tree analysis of HaMYB genes and Arabidopsis genes revealed that *HaMYB63* belongs to the same branch as *AtMYB116*, *AtMYB62*, *AtMYB112*, *AtMYB108*, *AtMYB2*, and *AtMYB78*, and it was shown that *AtMYB112* plays a positive role in the biosynthesis of anthocyanins in response to salt stress and high-light stress in Arabidopsis (Lotkowska *et al.*, 2015; Ampomah-Dwamena *et al.*, 2019). The expression level of the *AtMYB108* homologous gene *Hha072588* was significantly increased in *Hibiscus hamabo*, indicating that it plays an important role in resistance to drought stress (Wang *et al.*, 2021); Arabidopsis thaliana overexpressing the *AtMYB108*-homologous gene *RmMYB108* responds to freezing injury, salinity and water deficiency by increasing the activities of superoxide dismutase and peroxidase (Abubakar *et al.*, 2022), suggesting that HaMYB63 is involved in *H. ammodendron*'s response to abiotic stress. The results of subcellular localization of HaMYB63 show that it exists in the nucleus, and the results of transcriptional autoactivation show that it has transcriptional autoactivation activity, which suggests that HaMYB63 acts independently from other proteins when participating in the abiotic stress response of *H. ammodendron*, thus exerting its anti-stress functions.

Conclusions

In summary, 78 R2R3-MYB genes were identified at the *H. ammodendron* genome level, and bioinformatic analysis was performed on these genes. These genes were divided into 32 subgroups, the HaR2R3-MYB genes were randomly distributed on nine chromosomes, and three gene pairs were found to have undergone fragment duplication. *Cis*-acting elements of HaR2R3-MYB genes showed that the genes of this family are involved in hormonal pathways and environmental stresses, and under drought stress and salt stress, the expression of *HaMYB22*, *HaMYB27*, and *HaMYB42* was significantly changed. The transcriptome data showed that the expression of HaMYB63 changed significantly in different *H. ammodendron* samples, and it was high in the reddish-green and stiff assimilated branch samples. The subcellular localization of this protein showed that it was located in the nucleus, and the transcriptional self-activation results showed that it had transcriptional self-activation activity. The results of this study laid a foundation for understanding the function of R2R3-MYB genes and the molecular mechanism of resistance to stress in *H. ammodendron*.

Authors' Contributions

The experiments were created by HZ and ZL, who also edited the text. The tests were carried out by GZ, YR, and LM. Data was collected, downloaded, analyzed, and created figures and/or tables by GZ, CC, BW, and ZY. It was written by GZ, SL, MT, and YZ. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This work was supported by the Natural Science Fondation of Xinjiang Uygur Autonomous Rigion 2022D01D35.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Abubakar AS, Feng X, Gao G, Yu C, Chen J, Chen K, ... Zhu A (2022). Genome wide characterization of R2R3 MYB transcription factor from *Apocynum venetum* revealed potential stress tolerance and flavonoid biosynthesis genes. Genomics 114(2):110275. *https://doi.org/10.1016/j.ygeno.2022.110275*

Ambawat S, Sharma P, Yadav NR, Yadav RC (2013). MYB transcription factor genes as regulators for plant responses: an overview. Physiology and Molecular Biology of Plants 19:307-321. *https://doi.org/10.1007/s12298-013-0179-1*

Ampomah-Dwamena C, Thrimawithana AH, Dejnoprat S, Lewis D, Espley RV, Allan AC (2019). A kiwifruit (*Actinidia deliciosa*) R2R3-MYB transcription factor modulates chlorophyll and carotenoid accumulation. New Phytologist 221(1):309-325. *https://doi.org/10.1111/nph.15362*

Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, De Castro E, ... Stockinger H (2012). ExPASy: SIB bioinformatics resource portal. Nucleic Acids Research 40(W1):W597-W603. *https://doi.org/10.1093/nar/gks400*

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Noble WS (2009). MEME SUITE: tools for motif discovery and searching. Nucleic Acids Research 37:W202-W208. *https://doi.org/10.1093/nar/gkp335*

Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R (2020). TBtools: an integrative toolkit developed for interactive analyses of big biological data. Molecular Plant 13(8):1194-1202. *https://doi.org/10.1016/j.molp.2020.06.009*

Cui J, Qiu T, Li L, Cui S (2023). *De novo* full-length transcriptome analysis of two ecotypes of *Phragmites australis* (swamp reed and dune reed) provides new insights into the transcriptomic complexity of dune reed and its long-term adaptation to desert environments. BMC Genomics 24(1):1-23. *https://doi.org/10.1186/s12864-023-09271-y*

Du H, Feng BR, Yang SS, Huang YB, Tang YX (2012). The R2R3-MYB transcription factor gene family in maize. PloS One 7(6):e37463. *https://doi.org/10.1371/journal.pone.0037463*

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L (2010). MYB transcription factors in *Arabidopsis*. Trends in Plant Science 15(10):573-581. *https://doi.org/10.1016/j.tplants.2010.06.005*

Feng G, Burleigh JG, Braun EL, Mei W, Barbazuk WB (2017). Evolution of the 3R-MYB gene family in plants. Genome Biology and Evolution 9(4):1013-1029. *https://doi.org/10.1093/gbe/evx056*

Gao F, Zhao HX, Yao HP, Li CL, Chen H, Wang AH, ... Wu Q (2016). Identification, isolation and expression analysis of eight stress-related R2R3-MYB genes in tartary buckwheat (*Fagopyrum tataricum*). Plant Cell Reports 35:1385-1396. *https://doi.org/10.1007/s00299-016-1971-5*

Gao H, Lü X, Ren W, Sun Y, Zhao Q, Wang G, ... Zhang J (2020). *HaASR1* gene cloned from a desert shrub, *Haloxylon ammodendron*, confers drought tolerance in transgenic *Arabidopsis thaliana*. Environmental and Experimental Botany 180:104251. *https://doi.org/10.1016/j.envexpbot.2020.104251*

He AL, Niu SQ, Zhao Q, Li YS, Gou JY, Gao HJ, ... Zhang JL (2018). Induced salt tolerance of perennial ryegrass by a novel bacterium strain from the rhizosphere of a desert shrub *Haloxylon ammodendron*. International Journal of Molecular Sciences 19(2):469. *https://doi.org/10.3390/ijms19020469*

Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K (2007). WoLF PSORT: protein localization predictor. Nucleic Acids Research 35:W585-W587. *https://doi.org/10.1093/nar/gkm259*

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 Genomics 13:1-19. *https://doi.org/10.1186/1471-2164-13-544*

Kim D, Paggi JM, Park C, Bennett C, Salzberg SL (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nature Biotechnology 37(8):907-915. *https://doi.org/10.1038/s41587-019-0201-4*

Klempnauer KH, Gonda TJ, Bishop JM (1982). Nucleotide sequence of the retroviral leukemia gene *v-myb* and its cellular progenitor *c-myb*: the architecture of a transduced oncogene. Cell 31(2):453-463. *https://doi.org/10.1016/0092-8674(82)90138-6*

Li J, Chang H, Liu T, Zhang C (2019). The potential geographical distribution of Haloxylon across Central Asia under climate change in the 21st century. Agricultural and Forest Meteorology 275:243-254. *https://doi.org/10.1016/j.agrformet.2019.05.027*

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(4):402-408. *https://doi.org/10.1006/meth.2001.1262*

Lotkowska ME, Tohge T, Fernie AR, Xue GP, Balazadeh S, Mueller-Roeber B (2015). The *Arabidopsis* transcription factor MYB112 promotes anthocyanin formation during salinity and under high light stress. Plant physiology 169(3):1862-1880. *https://doi.org/10.1104/pp.15.00605*

Love MI, Huber W, Anders S (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15(12):1-21. *https://doi.org/10.1186/s13059-014-0550-8*

Lu S, Wang J, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, ... Marchler-Bauer A (2020). CDD/SPARCLE: the conserved domain database in 2020. Nucleic Acids Research 48(D1):D265-D268. *https://doi.org/10.1093/nar/gkz991*

Lü XP, Gao HJ, Zhang L, Wang YP, Shao KZ, Zhao Q, Zhang JL (2019). Dynamic responses of *Haloxylon ammodendron* to various degrees of simulated drought stress. Plant Physiology and Biochemistry 139:121-131. *https://doi.org/10.1016/j.plaphy.2019.03.019*

Lü XP, Shao KZ, Xu JY, Li JL, Ren W, Chen J, ... Zhang JL (2022). A heat shock transcription factor gene (*HaHSFA1*) from a desert shrub, *Haloxylon ammodendron*, elevates salt tolerance in *Arabidopsis thaliana*. Environmental and Experimental Botany 201:104954. *https://doi.org/10.1016/j.envexpbot.2022.104954*

Ma Q, Wang X, Chen F, Wei L, Zhang D, Jin H (2021). Carbon sequestration of sand-fixing plantation of *Haloxylon ammodendron* in Shiyang River Basin: storage, rate and potential. Global Ecology and Conservation 28:e01607. *https://doi.org/10.1016/j.gecco.2021.e01607*

Manna M, Thakur T, Chirom O, Mandlik R, Deshmukh R, Salvi P (2021). Transcription factors as key molecular target to strengthen the drought stress tolerance in plants. Physiologia Plantarum 172(2):847-868. *https://doi.org/10.1111/ppl.13268*

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 Biology 8:1-15. *https://doi.org/10.1186/1471-2229-8-83*

Paz-Ares J, Ghosal D, Wienand U, Peterson PA, 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. The EMBO Journal 6(12):3553-3558. *https://doi.org/10.1002/j.1460-2075.1987.tb02684.x*

Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nature Biotechnology 33(3):290-295. *https://doi.org/10.1038/nbt.3122*

Potter SC, Luciani A, Eddy SR, Park Y, Lopez R, Finn RD (2018). HMMER web server: 2018 update. Nucleic Acids Research 46(W1):W200-W204. *https://doi.org/10.1093/nar/gky448*

Romani F, Moreno JE (2021). Molecular mechanisms involved in functional macroevolution of plant transcription factors. New Phytologist 230(4):1345-1353. *https://doi.org/10.1111/nph.17161*

Rusconi F, Simeoni F, Francia P, Cominelli E, Conti L, Riboni M, ... Galbiati M (2013). The *Arabidopsis thaliana MYB60* promoter provides a tool for the spatio-temporal control of gene expression in stomatal guard cells. Journal of Experimental Botany 64(11):3361-3371. *https://doi.org/10.1093/jxb/ert180*

Salih H, Bai W, Zhao M, Liang Y, Yang R, Zhang D, Li X (2023). Genome-wide characterization and expression analysis of transcription factor families in desert moss *Syntrichia caninervis* under abiotic stresses. International Journal of Molecular Sciences 24(7):6137. *https://doi.org/10.3390/ijms24076137*

Shen XJ, Wang YY, Zhang YX, Guo W, Jiao YQ, Zhou XA (2018). Overexpression of the wild soybean R2R3-MYB transcription factor *GsMYB15* enhances resistance to salt stress and *Helicoverpa armigera* in transgenic *Arabidopsis*. International Journal of Molecular Sciences 19(12):3958. *https://doi.org/10.3390/ijms19123958*

Shin R, Burch AY, Huppert KA, Tiwari SB, Murphy AS, Guilfoyle TJ, Schachtman DP (2007). The *Arabidopsis* transcription factor MYB77 modulates auxin signal transduction. The Plant Cell 19(8):2440-2453. *https://doi.org/10.1105/tpc.107.050963*

Shriti S, Paul S, Das S (2023). Overexpression of CaMYB78 transcription factor enhances resistance response in chickpea against *Fusarium oxysporum* and negatively regulates anthocyanin biosynthetic pathway. Protoplasma 260(2):589-605. *https://doi.org/10.1007/s00709-022-01797-4*

Song J, Feng G, Tian CY, Zhang FS (2006). Osmotic adjustment traits of *Suaeda physophora*, *Haloxylon ammodendron* and *Haloxylon persicum* in field or controlled conditions. Plant Science 170(1):113-119. *https://doi.org/10.1016/j.plantsci.2005.08.004*

Stracke R, Holtgräwe D, Schneider J, Pucker B, Rosleff Sörensen T, Weisshaar B (2014). Genome-wide identification and characterisation of *R2R3-MYB* genes in sugar beet (*Beta vulgaris*). BMC Plant Biology 14(1):1-17. *https://doi.org/10.1186/s12870-014-0249-8*

Stracke R, Werber M, Weisshaar B (2001). The *R2R3-MYB* gene family in *Arabidopsis thaliana*. Current Opinion in Plant Biology 4(5):447-456. *https://doi.org/10.1016/S1369-5266(00)00199-0*

Subramanian B, Gao S, Lercher MJ, Hu S, Chen WH (2019). Evolview v3: a webserver for visualization, annotation, and management of phylogenetic trees. Nucleic Acids Research 47(W1):W270-W275. *https://doi.org/10.1093/nar/gkz357*

Sun J, Xu J, Qu W, Han X, Qiu C, Gai Z, ... Li Z (2023). Genome-wide analysis of R2R3-MYB transcription factors reveals their differential responses to drought stress and ABA treatment in desert poplar (*Populus euphratica*). Gene 855:147124. *https://doi.org/10.1016/j.gene.2022.147124*

Sun W, Ma Z, Chen H, Liu M (2019). MYB gene family in potato (*Solanum tuberosum* L.): genome-wide identification of hormone-responsive reveals their potential functions in growth and development. International Journal of Molecular Sciences 20(19):4847. *https://doi.org/10.3390/ijms20194847*

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30(12):2725-2729. *https://doi.org/10.1093/molbev/mst197*

Voorrips R (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. Journal of Heredity 93(1):77-78. *https://doi.org/10.1093/jhered/93.1.77*

Wang Y, Tang H, DeBarry JD, Tan X, Li J, Wang X, ... Paterson AH (2012). *MCScanX*: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. Nucleic Acids Research 40(7):e49-e49. *https://doi.org/10.1093/nar/gkr1293*

Wang Z, Ni L, Hua J, Liu L, Yin Y, Li H, Gu C (2021). Transcriptome analysis reveals regulatory framework for salt and drought tolerance in *Hibiscus hamabo* siebold & zuccarini. Forests 12(4):454. *https://doi.org/10.3390/f12040454*

Wyrzykowska A, Bielewicz D, Plewka P, Sołtys-Kalina D, Wasilewicz-Flis I, Marczewski W, ... Szweykowska-Kulinska Z (2022). The MYB33, MYB65, and MYB101 transcription factors affect *Arabidopsis* and potato responses to drought by regulating the ABA signaling pathway. Physiologia Plantarum 174(5):e13775. *https://doi.org/10.1111/ppl.13775*

Yang B, Li Y, Song Y, Wang X, Guo Q, Zhou L, ... Zhang C (2022). The R2R3-MYB transcription factor VcMYB4a inhibits lignin biosynthesis in blueberry (*Vaccinium corymbosum*). Tree Genetics & Genomes 18(3):27. *https://doi.org/10.1007/s11295-022-01560-z*

Yao T, Zhang J, Xie M, Yuan G, Tschaplinski TJ, Muchero W, Chen JG (2021). Transcriptional regulation of drought response in Arabidopsis and woody plants. Frontiers in Plant Science 11:572137. *https://doi.org/10.3389/fpls.2020.572137*

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13:1-11. *https://doi.org/10.1186/1471-2105-13-134*

Zhang S, Zhao Q, Zeng D, Xu J, Zhou H, Wang F, ... Li Y (2019). RhMYB108, an R2R3-MYB transcription factor, is involved in ethylene-and JA-induced petal senescence in rose plants. Horticulture Research 6:131. *https://doi.org/10.1038/s41438-019-0221-8*

Zhang, K, Su Y, Wang T, Liu T (2016). Soil properties and herbaceous characteristics in an age sequence of *Haloxylon ammodendron* plantations in an oasis-desert ecotone of northwestern China. Journal of Arid Land 8:960-972. *https://doi.org/10.1007/s40333-016-0096-6*

Zhao K, Cheng Z, Guo Q, Yao W, Liu H, Zhou B, Jiang T (2020). Characterization of the poplar R2R3-MYB gene family and over-expression of *PsnMYB108* confers salt tolerance in transgenic tobacco. Frontiers in Plant Science 11:571881. *https://doi.org/10.3389/fpls.2020.571881*

Zhou Z, Wei X, Lan H (2023). CgMYB1, an R2R3-MYB transcription factor, can alleviate abiotic stress in an annual halophyte *Chenopodium glaucum*. Plant Physiology and Biochemistry 196:484-496. *https://doi.org/10.1016/j.plaphy.2023.01.055*

Zhu L, Guan Y, Zhang Z, Song A, Chen S, Jiang J, Chen F (2020). *CmMYB8* encodes an R2R3 MYB transcription factor which represses lignin and flavonoid synthesis in chrysanthemum. Plant Physiology and Biochemistry 149:217-224. *https://doi.org/10.1016/j.plaphy.2020.02.010*

Zhu Y, Hu X, Wang P, Wang H, Ge X, Li F, Hou Y (2022). *GhODO1*, an R2R3-type MYB transcription factor, positively regulates cotton resistance to *Verticillium dahliae* via the lignin biosynthesis and jasmonic acid signaling pathway. International Journal of Biological Macromolecules 201:580-591. *https://doi.org/10.1016/j.ijbiomac.2022.01.120*