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

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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/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 as
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^{-ΔΔCt} 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 MgCl₂ 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 pGBKKT7 vector was used as the self-activating vector. The Nde I and Bam H I endonucleases (Thermo Fisher, USA) were selected to linearize the pGBKKT7 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-a-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.

![Chromosome localization map of the R2R3-MYB family of *H. ammodendron*](image)

**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* (SS28368.1) and *HaMYB17* (SS11433.1), *HaMYB44* (SS24322.1) and *HaMYB36* (SS19636.1), and *HaMYB42* (SS23511.1) and *HaMYB27* (SS15191.1), 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.
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.
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, and 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 $|\log_2\text{fold change}| \geq 1.5$ 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).

![Volcano plot of differentially expressed genes](image1)

**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 analyzed.

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 NcoI and SpeI 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 WolFPsORT (https://wolfpsort.hgc.jp/) online prediction results.

To analyze whether HaMYB63 has transcriptional self-activating activity, we constructed transcriptional self-activating vectors based on the CDS of HaMYB63 and the pGBK T7 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 pGBK7-HaMYB63 yeast could both grow on SD-Trp/-His/Ade-defective medium, the negative yeast could not grow normally, and the positive yeast and pGBK7-HaMYB63 yeast grown on SD-Trp/-His/Ade-defective medium containing X-a-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)

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Conflict of Interests

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

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