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Genome-wide identification, expression analysis, and stress response analysis of the *RdbZIP* gene family in *Rhododendron delavayi*

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Abstract

Background Basic leucine zipper (bZIP) gene family members represent one of the most diverse and largest groups of transcription factors in eukaryotes. Research has demonstrated that bZIP transcription factors play crucial roles not only in plant growth and development but also in response to various abiotic stresses. However, studies focusing on bZIP factors in *Rhododendron delavayi* (*RdbZIPs*) remain limited.

Result In this study, a total of 59 *RdbZIPs* were identified using bioinformatics approaches, and these could be classified into 13 subfamilies based on the genomic data of *R. delavayi*. Members of the same RdbZIP subfamily exhibited similar gene structures and conserved motifs, and were unevenly distributed across the 13 chromosomes of *R. delavayi*. Collinearity analysis revealed a total of 20 duplication events, comprising 3 pairs of tandem duplications and 17 pairs of segmental duplications. Additionally, *cis*-acting element analysis indicated that *RdbZIP* family members may be involved in various biological processes, including transcription, development, hormone regulation, and responses to biotic and abiotic stresses. Transcriptomic analysis revealed that *RdbZIP* family genes were highly expressed in reproductive tissues. RT-qPCR expression analysis revealed that many selected *RdbZIP* genes were significantly upregulated under high salinity and drought conditions, suggesting their potential involvement in stress-responsive regulatory networks.

Conclusion This study provides the first comprehensive characterization of the bZIP transcription factor family in *Rhododendron delavayi*, laying a foundational framework for functional studies of individual *RdbZIP* genes. The results highlight the pivotal role of *RdbZIP* genes in abiotic stress tolerance, which is crucial for understanding the adaptive mechanisms of *R. delavayi*. Future research should focus on the functional validation of key *RdbZIP* genes and elucidation of their regulatory pathways, which may contribute to the genetic improvement of *Rhododendron* species under adverse environmental conditions.

Clinical trial Not applicable.

Keywords Basic leucine zipper (*bZIP*) gene family, *Rhododendron delavayi*, Abiotic stresses, Gene expression

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Introduction

The bZIP transcription factor, fully known as the basic leucine zipper transcription factor, is a crucial type of transcriptional regulatory factor in eukaryotes [1–3]. This transcription factor is characterized by unique structural features, including a basic region at the N-terminus and a leucine zipper region at the C-terminus. The basic region is highly conserved and comprises approximately 16–20 basic amino acid residues, which form the nuclear localization sequence and DNA recognition domain, enabling specific binding to DNA sequences [1]. Its core binding sequence is typically ACGT. In contrast, the leucine zipper region is less conserved and consists of one or more heptapeptide repeat regions that form an α-helix. These heptapeptide repeat regions contain leucine or other hydrophobic residues, facilitating hydrophobic interactions between them [2, 4]. This interaction causes two bZIP transcription factors to wrap around each other, resulting in the formation of a dimer and a supercoiled structure.

Studies have shown that bZIP transcription factors play important roles in a diverse range of biological processes in organisms [5–9], including plant growth, development, and maturation of flowers. *AtbZIP9* and *AtbZIP46* are involved in vascular development, leaf regulation, floral organ number, and floral meristem characteristics in *Arabidopsis* [8, 9]. Additionally, bZIP transcription factors have been reported to respond to environmental stresses, including drought [10–15], high temperature [16], low temperature [17], and salt stress [18–20], thereby increasing plant stress resistance through the regulation of related gene expression. For example, in *Arabidopsis*, *AtbZIP10*, *AtbZIP25*, and *AtbZIP53* modulate seed germination by regulating auxin signal transduction, whereas *AtbZIP59* (PosF21) has been shown to facilitate auxin-induced callus formation and plant regeneration [13]. In rice (*Oryza sativa*), the *OsbZIP12* (*ABF1*) gene inhibits the expression of *OsEhd1*, resulting in delayed flowering in transgenic rice [14]. Under abiotic stress conditions, endogenous abscisic acid (ABA) levels in plant tissues rise sharply, triggering a suite of adaptive responses that are essential for maintaining both survival and productivity [21]. Central to this response are ABA-responsive elements (ABREs), *cis*-regulatory sequences defined by an ACGT core motif and located in the promoters of many stress-responsive and ABA-inducible genes [22]. bZIP transcription factors recognize and bind these ABREs, directly activating the transcription of downstream target genes and thereby enhancing the plant's tolerance to adverse environmental conditions [18]. *AtbZIP1* has been shown to bind specifically to ABRE sequences, modulating ABA signal transduction to confer greater stress resilience. *OsABF2* operates within the ABA signaling pathway. Its overexpression leads to markedly improved salt and drought tolerance. Similarly, overexpression of *OsbZIP23* increases ABA sensitivity in rice, further enhancing tolerance to both salinity and water deficit [15, 23]. In maize (*Zea mays*), ectopic expression of *ZmbZIP72* in transgenic *Arabidopsis* boosts ABA responsiveness, resulting in significantly elevated salt and drought tolerance [24]. In soybean (*Glycine max*), the *bZIP* genes *GmbZIP44*, *GmbZIP62*, *GmbZIP132* and *GmbZIP78*, when ectopically expressed in Arabidopsis, suppress ABA signaling and thereby confer enhanced salt tolerance [25, 26]. The overexpression of the *TabZIP15* gene in wheat (*Triticum aestivum*) also significantly improves salt tolerance [19]. In peanuts (*Arachis hypogaea*), the *bZIP* G subfamily genes *AdbZIP15*, *AdbZIP17*, and *AdbZIP19* may contribute to salt stress tolerance [20]. Recently, a genome-wide bZIP transcription factor family has been identified not only in model plants such as *Arabidopsis* [13], rice [4], and maize [27] but also in various economic and horticultural plants such as rapeseed [28], soybean [29], watermelon [30], and poplar [31].

Rhododendron delavayi is an evergreen shrub or small tree belonging to the genus *Rhododendron* in the family Ericaceae. It is widely distributed in evergreen broad-leaved forests and shrubs at altitudes ranging from 1,200 to 3,200 m, serving as an important component of alpine woodlands [32]. This species has significant ornamental and medicinal value [33]. Owing to the increasing frequency of extreme weather events in recent years, *R. delavayi* now faces severe drought stress, threatening the stability of its natural populations. Although research on *Rhododendron* has primarily addressed genetic evolution, ornamental trait development, and genome assembly, the *bZIP* transcription factor family in *R. delavayi* remains unexplored. The recent availability of high-quality genomic and transcriptomic datasets, combined with the species' ecological significance and remarkable environmental adaptability, provides an ideal foundation for a systematic analysis of this gene family.

Materials and methods

Plant materials

Seeds of *R. delavayi* were collected from the Baili Rhododendron Nature Reserve in Guizhou, China (105°45′45″E, 27°08′30″N). Permission for seed collection was granted by the management authority of the Reserve, and the collection was conducted in accordance with the relevant local regulations. These seeds were then germinated in an incubator at 32 °C for two weeks. Following germination, the seedlings were cultured in pots measuring 10 cm in length and 10 cm in width, maintained in an incubator under a 16/8 h light/dark cycle at a temperature of 22 °C, and with a relative humidity of 40% for approximately 120 days. 1/2 Hoagland nutrient solution was applied to

the seedlings weekly until the seventh leaf was generated. Subsequently, seedlings of similar sizes were selected for salt and drought treatments. For the salt treatment, *R. delavayi* seedlings were exposed to a 150 mM NaCl solution, which was prepared by dissolving NaCl in a 1/2 Hoagland solution. For the drought treatment, the seedlings were subjected to 20% PEG 6000 for durations of 0, 3, 6, 12, and 24 h, whereas the control group received 1/2 Hoagland solution. More than three replicates were established for each treatment.

Identification and bioinformatic analysis of *bZIPs* in *R. delavayi* (*RdbZIPs*)

The *bZIP* members were identified from the published genome of *R. delavayi*, which was downloaded from the Rhododendron Plant Genome Database (http://bioinfor.kib.ac.cn/RPGD/. Accessed 21 Sept 2024) [33]. To identify the putative bZIP TF members in *R. delavayi*, two methodologies were employed: Hidden Markov Models (HMMs) and BLASTp (Basic Local Alignment Search Tool for proteins) [34]. Putative genes were searched via HMMER 3.0 software with a cutoff of e value $\leq 10^{-5}$ on the basis of the HMM file of the bZIP domain (PF00170) obtained from Pfam (https://www.ebi.ac.uk/interpro/. Accessed 21 Sept 2024) [35, 36]. Concurrently, the bZIP protein sequences from *Arabidopsis* (*At-bZIPs*) were downloaded from The *Arabidopsis* Information Resource (TAIR, https://www.arabidopsis.org/) and subjected to a local BLASTp search against the *R. delavayi* genome, with an e-value threshold of $\leq 10^{-5}$. The results from both methods were subsequently merged to determine the *RdbZIPs*. Finally, to confirm that these sequences contained the complete NAM domain, the *RdbZIPs* were further validated via an NCBI batch CD search (https://www.ncbi.nlm.nih.gov/cdd. Accessed 22 Sept 2024) with an e-value parameter of $\leq 10^{-5}$, as well as the SMART (Simple Modular Architecture Research Tool. http://smart.embl-heidelberg.de/. Accessed 22 Sept 2024) database [37].

The chemical and physical characteristics of the RdbZIP proteins, including the number of amino acids, molecular weight (MW), and isoelectric point (pI), were assessed via ExPASy (https://www.expasy.org/. Accessed 23 Sept 2024) with default parameters [38]. Additionally, the subcellular localization of *RdbZIPs* was predicted via CELLO v.2.5 (http://cello.life.nctu.edu.tw/. Accessed 23 Sept 2024).

Phylogenetic analysis of *RdbZIPs* and *AtbZIPs* sequences

The MUSCLE program was employed to align the amino acid sequences of *RdbZIPs* and *AtbZIPs* via the L-INS-i algorithm [39]. Phylogenetic trees were subsequently constructed via MEGA 11 v11.0.13 software, applying the Maximum Likelihood (ML) method in conjunction with the Tamura–Nei nucleotide substitution model [40]. The resulting phylogenetic tree was visualized via the Interactive Tree of Life (iTOL) platform (https://itol.embl.de/. Accessed 23 Sept 2024).

Gene duplication, collinearity, and evolutionary analysis of *RdbZIPs*

To predict gene duplication events among the *RdbZIPs*, the DupGen_finder tool was employed. Collinear gene pairs of *RdbZIPs* were subsequently identified using the JCVI tool with default parameters [41]. To evaluate the repetitive gene pairs among *RdbZIPs*, PRANK was utilized. The tandem duplication genes selected in this study have a sequence similarity of ≥ 80% and are located within 10 kb of each other, while the segmental duplication genes have a sequence similarity of ≥ 90% and are separated by distances greater than 10 kb. Finally, the KaKs_Calculator 2.0 was used to compute the values of Ka, Ks, and the Ka/Ks ratio, applying the NG model [42].

Analysis of *RdbZIPs* structure and conserved motifs

The gene structures of *RdbZIPs* were analyzed via GSDS 2.0 software (Gene Structure Display Server 2.0; http://gsds.cbi.pku.edu.cn//index.php. Accessed 23 Sept 2024) with default parameters [43]. To identify the conserved motifs of *RdbZIPs*, the MEME online tool (https://meme-suite.org/meme/index.html. Accessed 21 Sept 2024) was employed with the following parameters: an e-value parameter of ≤ 10 − 5 and a range of motif widths from 6 to 50. Furthermore, the conserved domain sequences of the *RdbZIPs* family were determined via the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/cdd/. Accessed 22 Sept 2024).

The analysis of chromosomal localization and *cis*-acting elements (CREs) in the promoter regions of *RdbZIPs* involves several steps [44]. First, the physical distribution data of *RdbZIPs* were obtained from the published genome annotation files of *R. delavayi*. The chromosomal localization of *RdbZIPs* was then visualized using TBtools. The 2000 bp upstream sequences of the promoter regions for each *RdbZIP* were extracted via TBtools and subsequently analyzed for the prediction of *cis*-elements using the online database PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/. Accessed 22 Sept 2024).

Gene expression patterns of *RdbZIPs* in different tissues and at different developmental stages

To analyze the expression of *RdbZIP* family members in various tissues of *R. delavayi*, transcript data from different tissues, including spotted petals, unspotted petals, spotted throat, unspotted throat, branchlet cortex, and leaf tissues, were obtained from the National Center for Biotechnology Information (NCBI) under the dataset

PRJNA907866 (https://www.ncbi.nlm.nih.gov/. Accessed on 23 Sept 2024) [45]. The expression levels of *RdbZIPs* were calculated via HISAT2 version 2.0.5 with default parameters [46], and TBtools was employed to generate a heatmap of *RdbZIP* gene expression.

RNA extraction and qRT–PCR analysis

The leaves from these seedlings were cryopreserved in liquid nitrogen, after which total RNA was extracted from the samples via a commercial RNA extraction kit (Aidlab Biotech, Beijing, China). The primers for the selected *RdbZIPs* were designed using Primer 5 software (Table S6). To prepare the cDNA library, we adhered to the TruSeq RNA Sample Prep v2 protocol (Illumina, USA), ensuring that the RNA RIN values were ≥ 8.0. The expression levels of *RdbZIPs* under salt and PEG treatment were assessed via quantitative reverse transcription PCR (qRT−PCR). The relative expression of the genes at each time point was calculated via the $2^{-\Delta\Delta Ct}$ method [47]. The reaction protocol included an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. All experiments were conducted with three independent biological replicates, each consisting of three technical replicates.

Statistical analysis

All experiments were conducted with a minimum of three technical replicates or three biological replicates. Data analyses were carried out via IBM SPSS Statistics 26 and GraphPad Prism software. Differences among the various groups were assessed via the least significant difference (LSD) test, with a significance threshold set at $p < 0.05$.

Results

Identification, chemical and physical features of the RdbZIPs proteins

After excluding the redundant genes, a total of 59 RdbZIPs transcription factors were screened in the *R. delavayi* genome via protein sequence alignment and conserved domain screening (Table S1). On the basis of the bioinformatics results, all the RdbZIPs are hydrophilic proteins, with average hydropathicity values ranging from – 1.086 to -0.245. These RdbZIPs encode protein lengths ranging from 99 to 1016 amino acids and molecular weights ranging from 11.48 (RdbZIP41) to 111.54 kDa (RdbZIP18). The results of the subcellular localization prediction revealed that a majority of the RdbZIPs were localized only in the nucleus, whereas only four RdbZIPs were localized in the mitochondrial matrix, chloroplasts, or vacuoles.

Phylogenetic analysis and chromosomal distribution of *RdbZIPs*

To determine the evolutionary relationships and classification of the RdbZIPs, these AtbZIPs (78 bZIP members in total) were used together with the RdbZIPs to construct a phylogenetic tree via MEGA software. The results revealed that these RdbZIPs and AtbZIPs can be divided into 13 subfamilies (subfamilies A, B, C, D, E, F, G, H, I, J, K, S, and M) with gene numbers ranging from two to 27 based on their relationships with AtbZIPs (Fig. 1). Among these subfamilies, subfamily S was the largest group with 10 RdbZIPs and 17 AtbZIPs, followed by subfamily A (10 RdbZIPs and 13 AtbZIPs), subfamily I (7 RdbZIPs and 12 AtbZIPs), and subfamily D (7 RdbZIPs and 10 AtbZIPs), while only one RdbZIP was identified in subfamily B (four genes), subfamily H (three genes), subfamily K (two genes), and subfamily M (two genes). Additionally, we noted that subfamilies A and S mainly consisted of genes with short sequences, whereas subfamilies G and D predominantly harbored long-sequence genes (Table S1).

Based on the *R. delavayi* genome annotation information, the chromosome locations of these *RdbZIPs* were determined. All of the *RdbZIPs* were successfully located on the corresponding chromosome (Fig. 2). These *RdbZIPs* were not evenly distributed across the 13 chromosomes, ranging from 1 gene on chromosome 02 to 12 to 10 genes on chromosome 07. These *RdbZIPs* are prone to be located in chromosome regions with high gene density. Moreover, some *RdbZIPs* genes shared similar chromosome locations and clustered into close phylogenetic branches (Fig. 2), suggesting that these genes were derived from tandem duplications (TDs).

Gene collinearity and evolution analysis of the *RdbZIPs*

Gene duplication events, including dispersed duplication (DSD) and TD events, are believed to be vital driving forces for the expansion of gene families. To identify gene duplication events, we detected intraspecific collinear gene pairs among these *bZIPs* in *R. delavayi* via JCVI v1.3.5 and DupGen _ finder. In addition, intraspecific collinear gene pairs between *R. delavayi* and *A. thaliana* and between *R. delavayi* and *Rosa rugosa* were also detected. The results revealed that a total of 19 duplicated gene pairs were found in these *RdbZIPs*, including an overrepresented whole-genome duplication (WGD) or segmental duplication (SD) incident (89.4%, 17 of 19 pairs) and three pairs of tandem duplication incidents (Fig. 3A, Table S2). This result indicated that DSD events were a great driving force for the *bZIPs* expansion in *R. delavayi*. Additionally, 50 and 57 interspecific collinear-gene pairs were detected in the pair of *R. delavayi* and *A. thaliana* and the pair of *R. delavayi* and *Rosa rugosa*, respectively (Fig. 3B, Table S3).

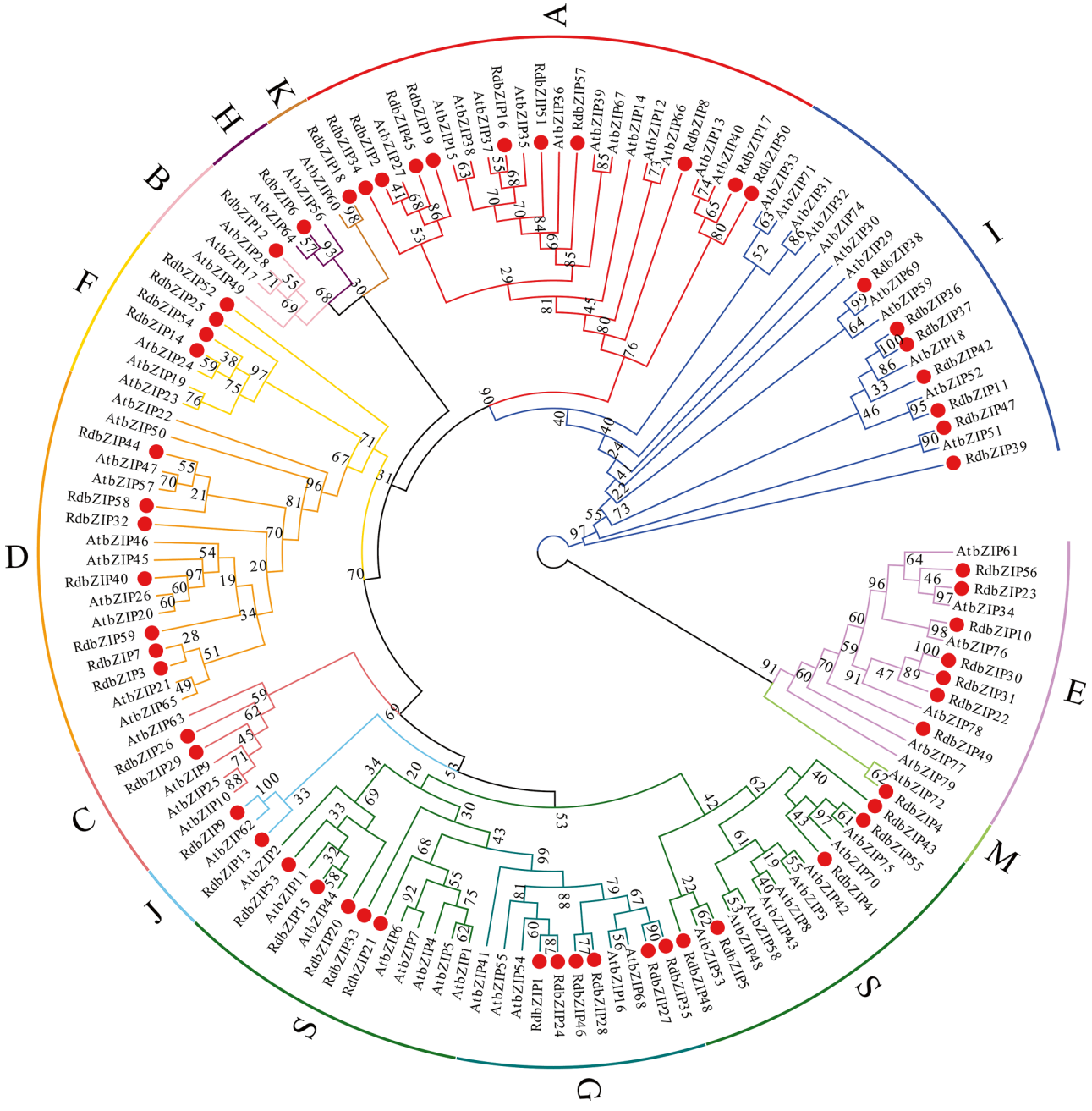

Fig. 1 Phylogenetic analysis of the bZIP family. The analysis involved 137 amino acid sequences from various plants, including *A. thaliana* (At) *R. delavayi* (Rd)

To evaluate the rate of gene divergence of *bZIPs* in *R. delavayi*, we used the Ka/Ks ratio, which is generally used to determine whether there are selective pressures on protein-coding genes. First, the Ka/Ks values of intraspecific *RdbZIPs* (17 pairs in total) were obtained (Fig. 4, Table S4). After excluding the genes with Ka or Ks values of 0, as these values indicate a lack of variation or synonymous substitutions, the average Ka/Ks ratio of the genes was 0.33. The Ka/Ks values of the interspecies (36 pairs) were subsequently calculated between *R. delavayi* and *A. arabidopsis*. Similarly, the values of Ka or Ks of 0 were removed. The Ka/Ks values of the *RdbZIPs* varied from 0.01 to 1.54 across the different species, and the majority of these pairs (44 of 53 in total) were distributed in the range of 0.1–0.3, whereas only one pair (*RsbZIP30* vs. *RsbZIP31*) was greater than 1. These results implied that most *RdbZIPs* were subjected to strong purifying selection pressures.

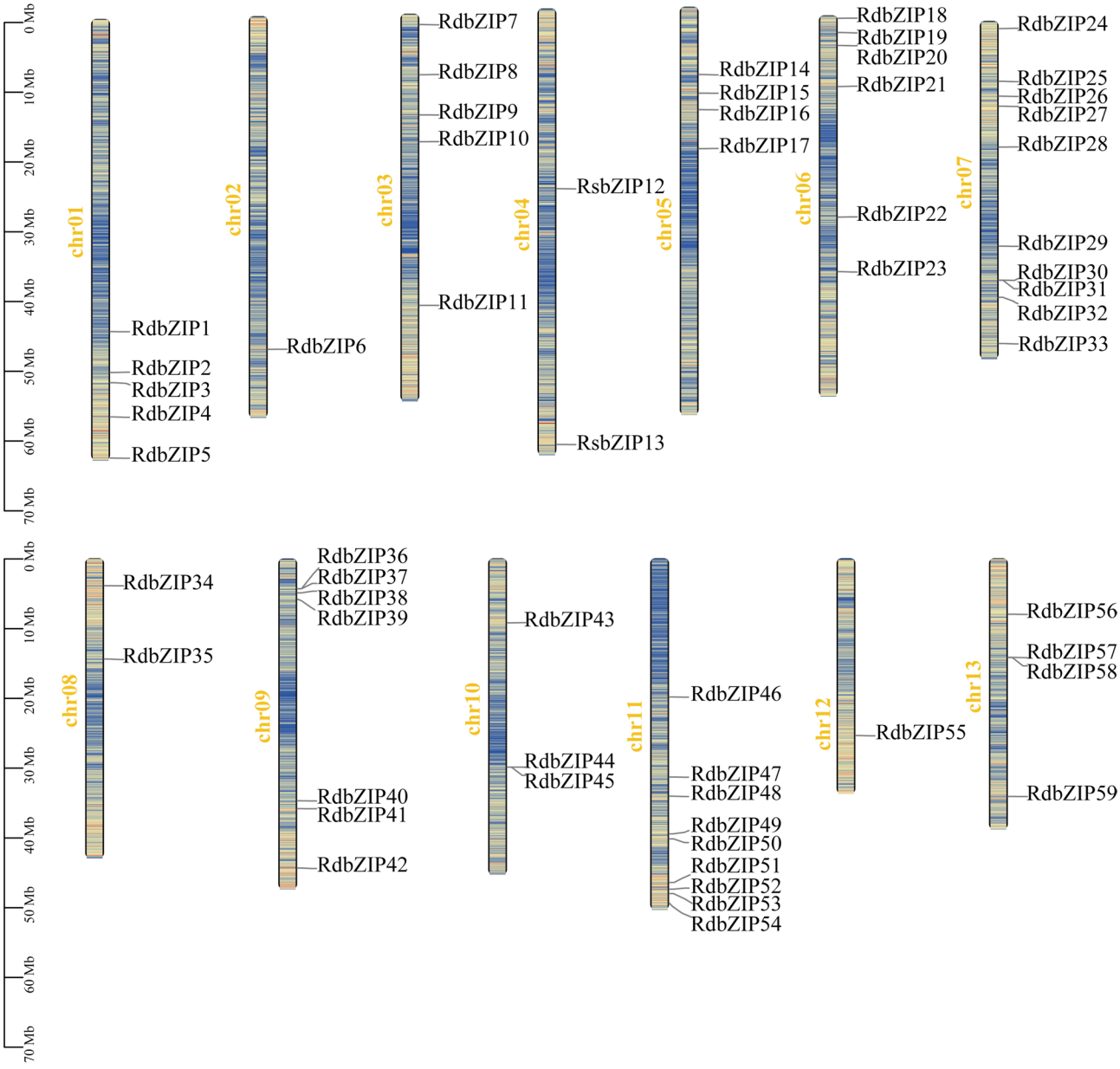

Fig. 2 The distribution of *RdbZIPs* on chromosomes in *R. delavayi*. The yellow font indicates the *R. delavayi* chromosome number, the black short line displays the names of the *RdbZIPs*, the scale on the left represents the length of each chromosome (Mb), and the blue to red gradient represents gene density of low to high

Conserved motif and exon–intron structure analysis of *RdbZIPs*

To determine the conserved motifs and exon–intron structure evolution of the *RdbZIP* family, we used MEME and MEGA7 software to analyze these *RdbZIPs*. The protein structure results indicated that the RdbZIP transcription factors consisted of 20 conserved motifs (Motif 1 to Motif 20) (Fig. 5). All the *RdbZIPs* contained Motif 1; similarly, with the exception of the genes in subfamily D, *RdbZIP12*, *RdbZIP18*, *RdbZIP22*, *RdbZIP45*, and *RdbZIP52*, all the other motifs included Motif 14. These findings suggest that Motif 1 and Motif 14 are relatively conserved in *RdbZIPs*. The *bZIP* family domain comprises two regions with distinct functions, leading to the speculation that Motif 1 may constitute one *bZIP* domain, whereas Motif 14 may represent a component of the other *bZIP* domain. In general, the motif constitution in different subfamilies was largely variable. For example, the genes in the A subfamily mainly consisted of five motifs, including Motifs 14, 15, 13, 1 and 5; the D subfamily mainly included Motifs 1, 8, 7, 2, 5, and 6; the G subfamily contained Motifs 12, 10, 1 and 14; and the K subfamily only included Motif 1 (Fig. 5B). Furthermore, *RdbZIP* has a range of 0 to 22 introns and 1 to 23

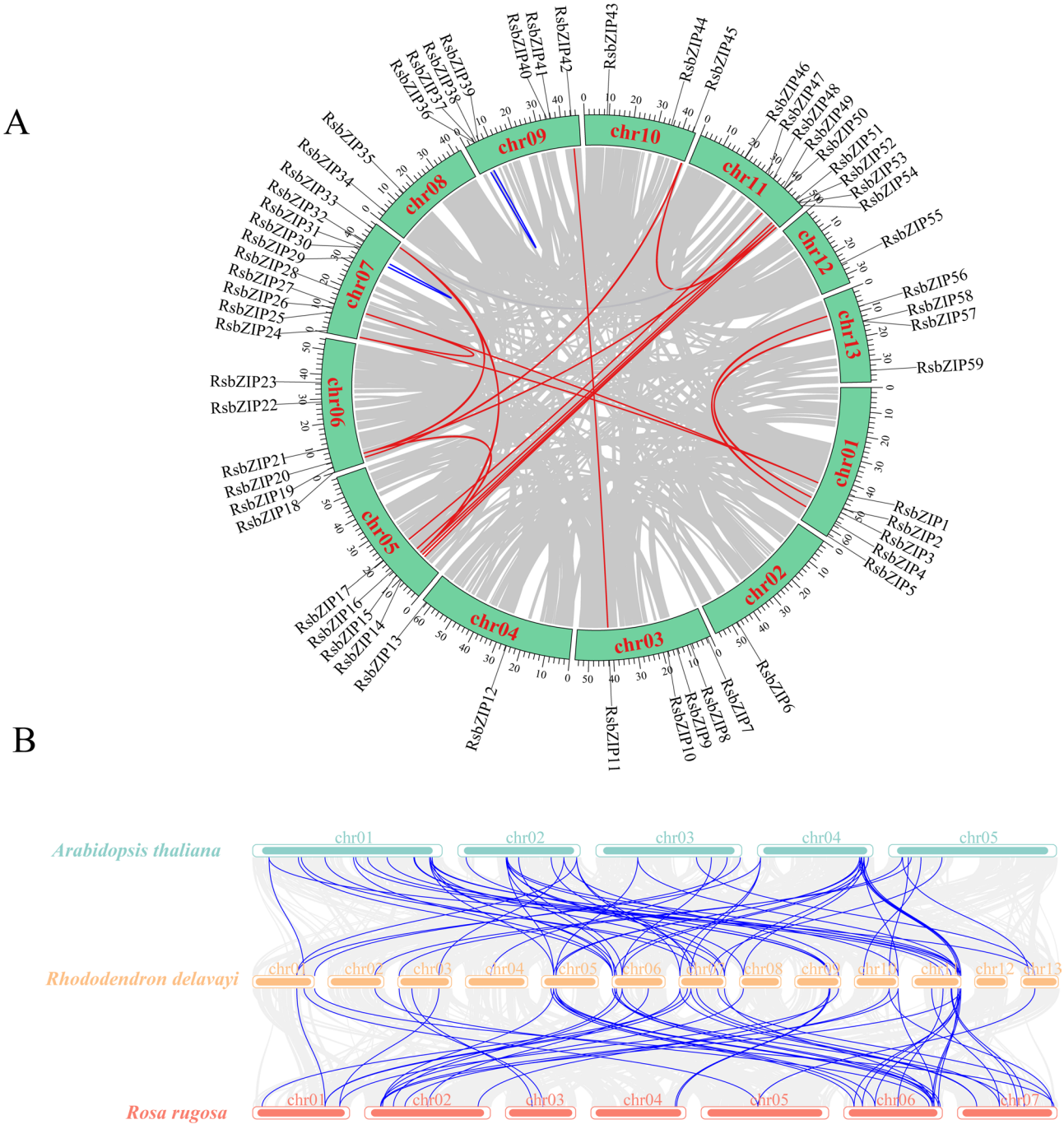

Fig. 3 Gene replication and synteny analysis of *RdbZIPs*. (**A**) The intraspecific collinear-gene pairs of *RdbZIPs* in *R. delavayi*. Red lines indicate segmental duplications, while blue lines represent tandem duplications. (**B**) The interspecific collinear-gene pairs in the pair of *R. delavayi* and *A. thaliana* and the pair of *R. delavayi* and *Rosa rugosa*

exons (Fig. 5C). Members of the same subfamily present similar numbers of exons and introns; however, notable structural differences exist among different subfamilies. For example, the genes of the S subfamily are relatively short, containing only 1 exon and 0 to 1 intron, whereas the two genes of the K subfamily possess 23 exons, resulting in relatively longer genes. These observations indicate close evolutionary relationships and highly similar conserved sequences within the same subfamily, suggesting that RdbZIP transcription factors of the same subfamily often share similar functions.

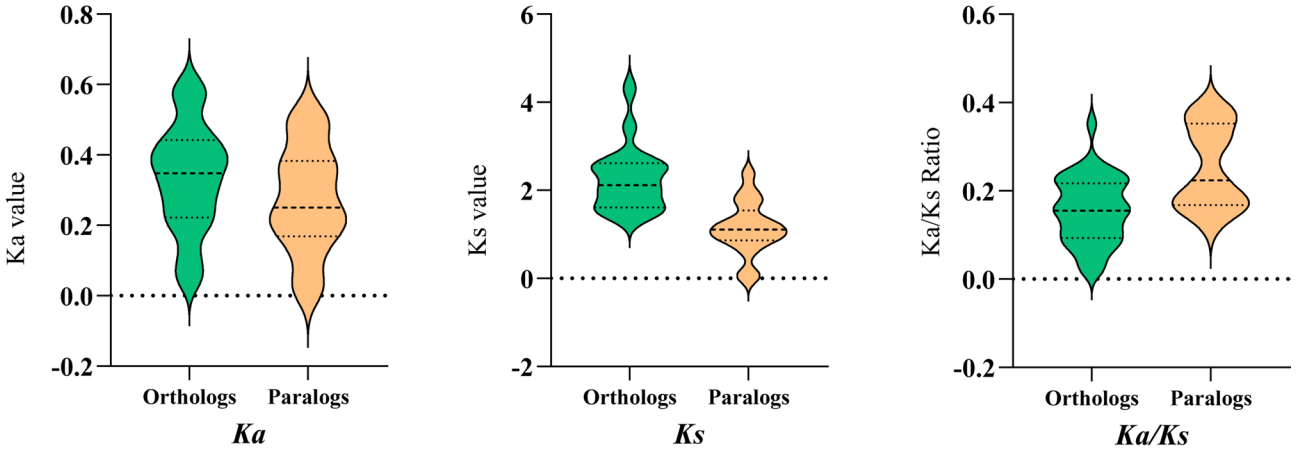

Fig. 4 The Ka, Ks, and Ka/Ks values of the *bZIP* genes in *R. delavayi* compared to *A. thaliana*

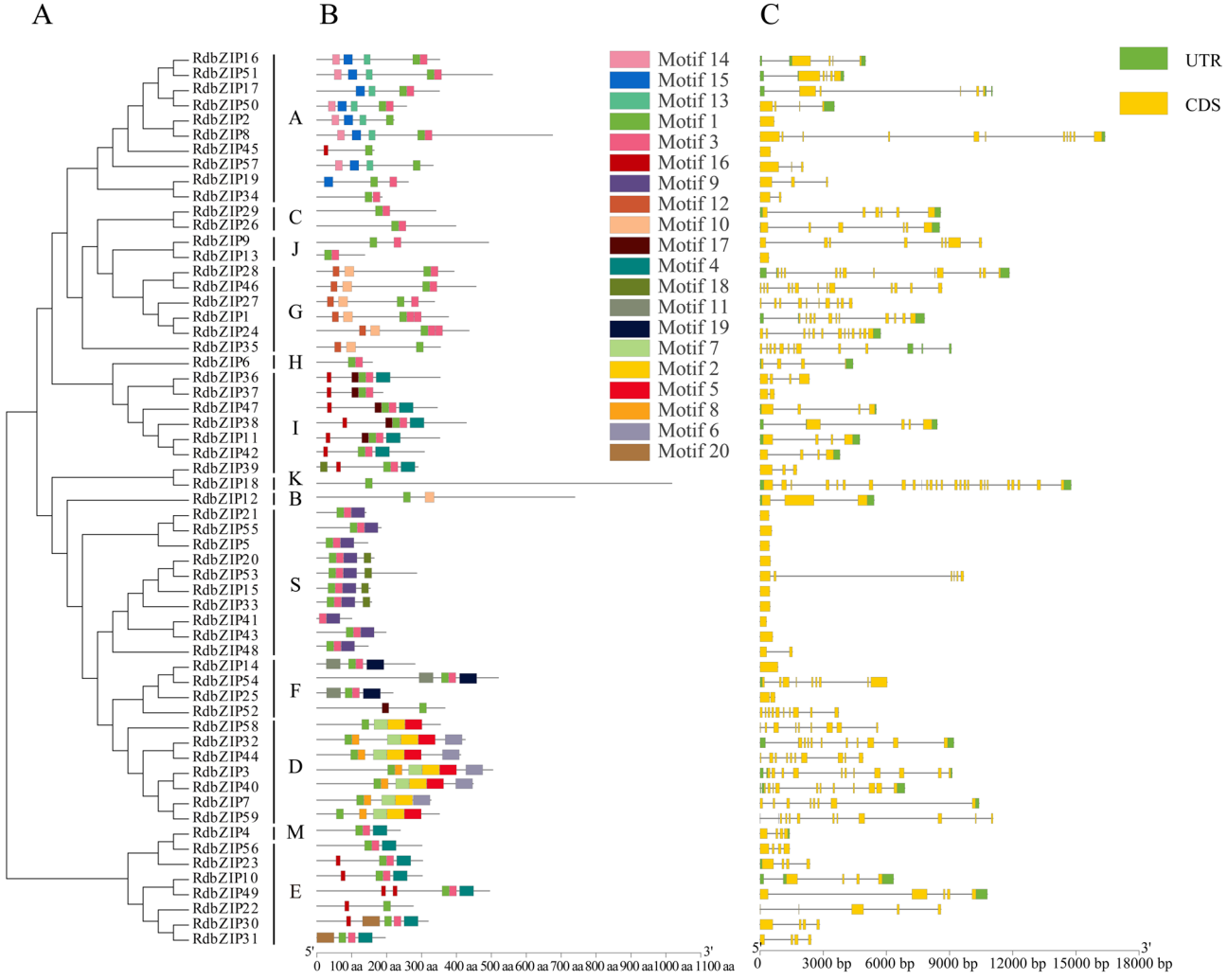

Fig. 5 Conserved motifs and exon-intron structure analysis of the *bZIP* gene in *Rhododendrons*. (**A**) Phylogenetic tree of RdbZIPs. The tree was generated using MEGA7 through the Maximum Likelihood method based on the protein sequences of RdbZIPs. (**B**) Conserved motif analysis. Eleven motifs were identified, with different colored boxes representing various types of motifs. (**C**) Exon-intron structures

Cis-acting element analysis of *RdbZIPs* and their functional prediction

Gene transcription regulation is typically achieved through the binding of different transcription factors to *cis*-acting elements in the promoter. To explore the *cis*-acting elements in the promoters of *RdbZIPs* in response to various environmental signals, a 2 kb promoter region of 49 genes was extracted and analyzed via the PlantCARE database (Fig. 6, Table S5). The results revealed a total of 1,544 *cis*-acting elements, with an average of 26.17 elements per gene. Among these *cis*-acting elements, five types of *cis*-elements (MBS, STRE, ARE, WUN-motif, and MRE; 458 elements in total) associated with the stress response; eight types responsible for the phytohormone response (ABRE, CGTCA-motif, TGACG-motif, ERE, TGA-element, TCA-element, GARF-motif, and P-box; 565 elements); and nine types (BOX-4, G-box, GATA-motif, ATCG-motif, GT1-motif, LTR, TC-rich repeats, TCT-motif, and CAT-box; 521 elements) responsible for growth development were detected. Additionally, more than 89.83% of the promoters (53 *RdbZIPs*) of *RdbZIPs* contained AREs, G-box, ABRE, Box 4, CGTCA-motif, TGACG-motif, or GT1-motif.

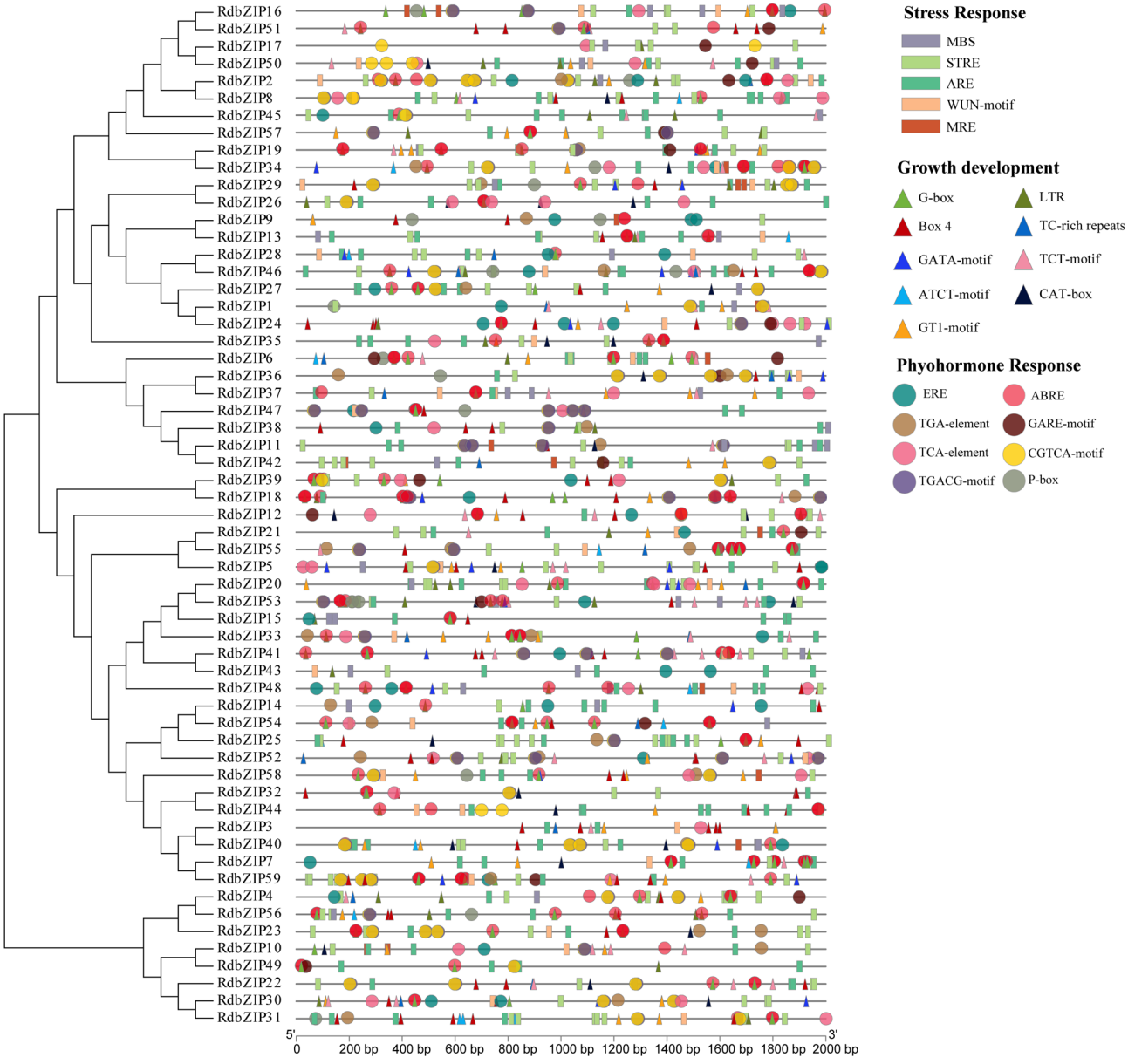

Fig. 6 *Cis*-element number analysis in the *RdbZIPs* gene family

Gene expression patterns of *RdbZIPs* in different tissues and at different developmental stages

To elucidate the expression patterns of the *RdbZIP* family, RNA-seq data from six tissues of *R. delavayi*, including spotted petals, unspotted petals, spotted throat, unspotted throat, branchlet cortex, and leaf, were utilized to generate a transcription pattern diagram for the *RdbZIPs* (Fig. 7). In general, nearly half of the *RdbZIPs* presented low expression levels across the six tissues (FPKM < 2). Furthermore, compared with those in the other five tissues, the expression levels of most *RdbZIPs* in leaves are consistently significantly lower. In particular, *RdbZIPs* are highly expressed in the other five tissues, whereas their expression levels in leaves are negligible.

Gene expression profiles of *RdbZIPs* in response to salt and drought stress

To further verify the potential functions of the *RdbZIPs* in response to stress, qRT−PCR analysis of 11 randomly selected *RdbZIPs* and one actin gene (Table S6) under high-salt (150 mM NaCl) and PEG treatments (simulating drought stress) was performed. With respect to the NaCl high-salt treatment, all of the selected *RdbZIPs* were significantly induced except *RdbZIP28* (Fig. 8A). Furthermore, the expression of these *RdbZIPs* generally reached the highest level after exposure to salt stress for 3–6 h and then decreased with increasing treatment time. However, compared with those under the high-salt treatment, the gene expression levels of these *RdbZIPs* were largely variable under the PEG treatment (Fig. 8B). For example, the expression levels of *RdbZIP4*, *RdbZIP9*, *RdbZIP47*, and *RdbZIP52* increased with prolonging treatment time. In contrast, *RdbZIP9* reached its maximum expression after 3 h of treatment, after which it subsequently decreased as the treatment continued. The expression levels of *RdbZIP20*, *RdbZIP43*, and *RdbZIP52* initially increased but then decreased, peaking at 6 h of treatment. Additionally, the *RdbZIP3* expression reached its maximum after 3 h, decreased, and then peaked again after 24 h. We noted that the expression levels of *RdbZIP47* exhibited opposing patterns across the two treatments, suggesting that the function of this gene varies in response to different stresses.

Discussion

The *bZIP* gene family plays crucial roles in regulating plant growth, development, and responses to biotic and abiotic stresses, including high temperature, drought, salt, cold, and ABA [5–9]. To date, the *bZIP* gene family has been characterized in various plant species, including model plants and economic and horticultural plants [13, 27–31]. However, there have been no reports on the *bZIP* gene family in *R. delavayi*. In this study, a total of 59 *RdbZIP* gene family members were identified *in R. delavayi*, a number comparable to the *bZIP* family members found in grape (*Vitis vinifera*) with 55 members [48], litchi (*Litchi chinensis*) with 54 members [49], and wax gourd (*Benincasa hispida*) with 61 members [50] but significantly lower than those in *Arabidopsis* (78 members) [13] and poplar (81 members) [31], although *R. delavayi* has a relatively close relationship with the latter two [51]. Previous studies have demonstrated that tandem duplication and large-scale segmental duplication contribute to the expansion of gene families during genome duplication [52–54]. Poplars have undergone at least three rounds of whole-genome duplication, followed by multiple segmental duplications, tandem duplications and transposition events [52]. Like poplar, a genome-triplication incident was also widespread in Brassicaceae species represented by *Arabidopsis* [53]. However, only one case of whole-genome duplication was detected in the *R. delavayi* genome [54], suggesting that whole-genome duplication is the key driving force for the expansion of gene families. In addition, phylogenetic analysis revealed that the *RdbZIP* family can be categorized into 12 distinct subfamilies. The distribution of *bZIP* gene family members in *R. delavayi*, ranked from highest to lowest, is as follows: A, D, S, B, and H. A similar distribution pattern has also been observed in *Arabidopsis* [13] and rice [4], indicating a certain degree of conservation of the *bZIP* subfamily across plant species.

Previous studies have shown that gene families typically undergo tandem duplication or large-scale segmental duplication events [55]. Given the relatively large number of members in the *bZIP* gene family, the present study analyzed tandem and segmental duplication events of *RdbZIP* genes in rhododendron. The results revealed that WGD and segmental duplication are the primary drivers of *RdbZIP* family expansion and evolution. In total, 17 pairs of segmental duplications and 2 pairs of tandem duplications were detected in *RdbZIP*, with duplications in subgroups A and S being most abundant, indicating that the increase in *RdbZIP* member number is chiefly attributable to segmental duplication (Fig. 3A, Table S3). Furthermore, Ka and Ks substitution rates and their ratio are commonly used to assess selection pressure on protein-coding genes [56]. Calculated Ka/Ks ratios for all duplicated *RdbZIP* gene pairs, with the sole exception of one tandem pair, were less than 1, indicating that these genes have been subject to strong purifying selection during evolution, with limited functional divergence (Fig. 4, Table S4). The only exception was the tandemly duplicated pair *RdbZIP30*/*RdbZIP31*, whose Ka/Ks ratio exceeded 1, suggesting that this pair may have undergone positive selection and adaptive evolution. In the collinearity analysis with the bZIP genes of the model plant *A. thaliana* and rose (*Rosa chinensis*), numerous colinear genes were identified (Fig. 3B). However, collinearity

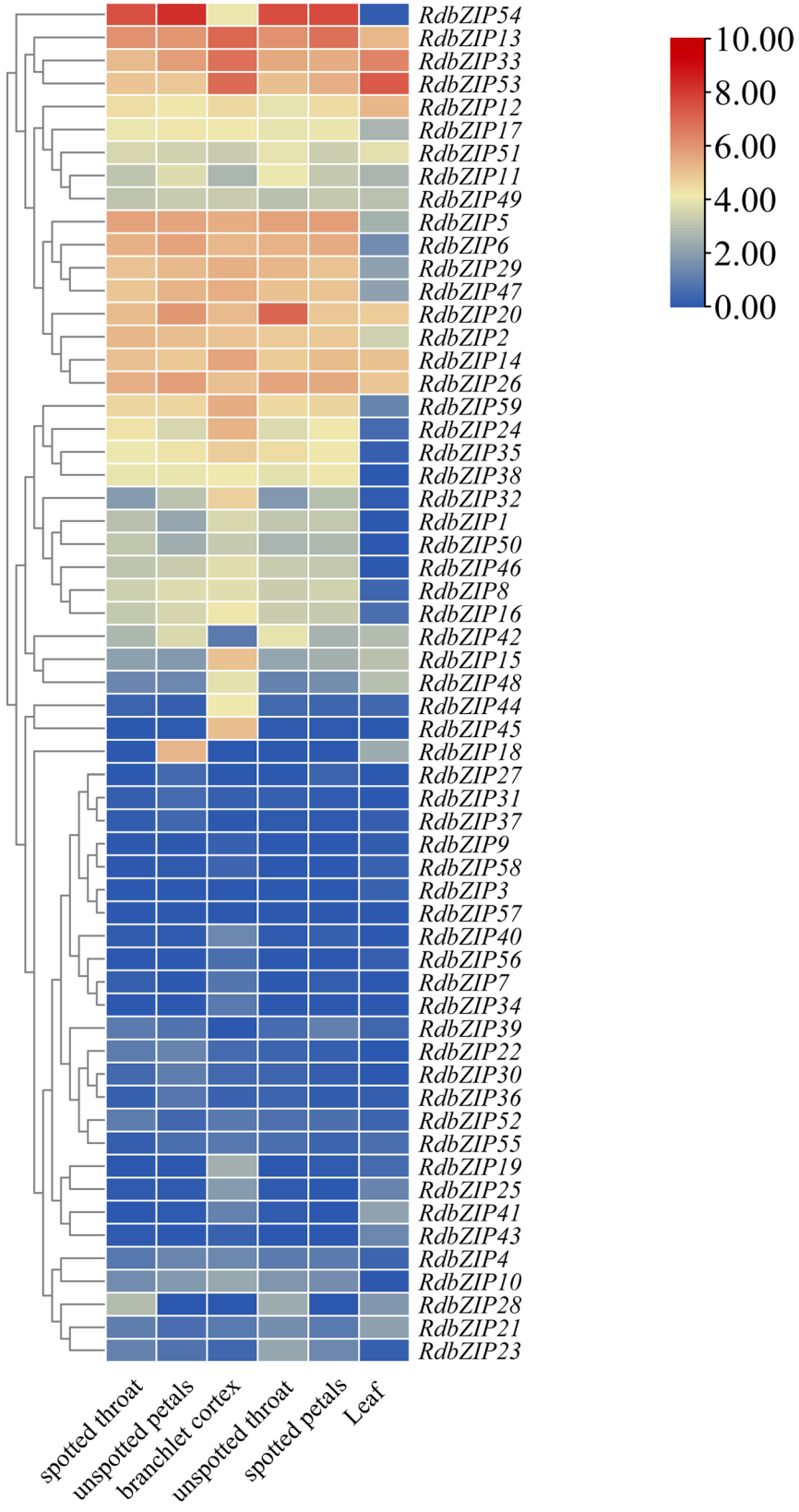

Fig. 7 The expression profiles of *RdbZIP* genes in different tissues and developmental stages. Gene expression levels in the heatmap are shown as $\log_2$(FPKM + 1), with red indicating high expression and blue indicating low expression

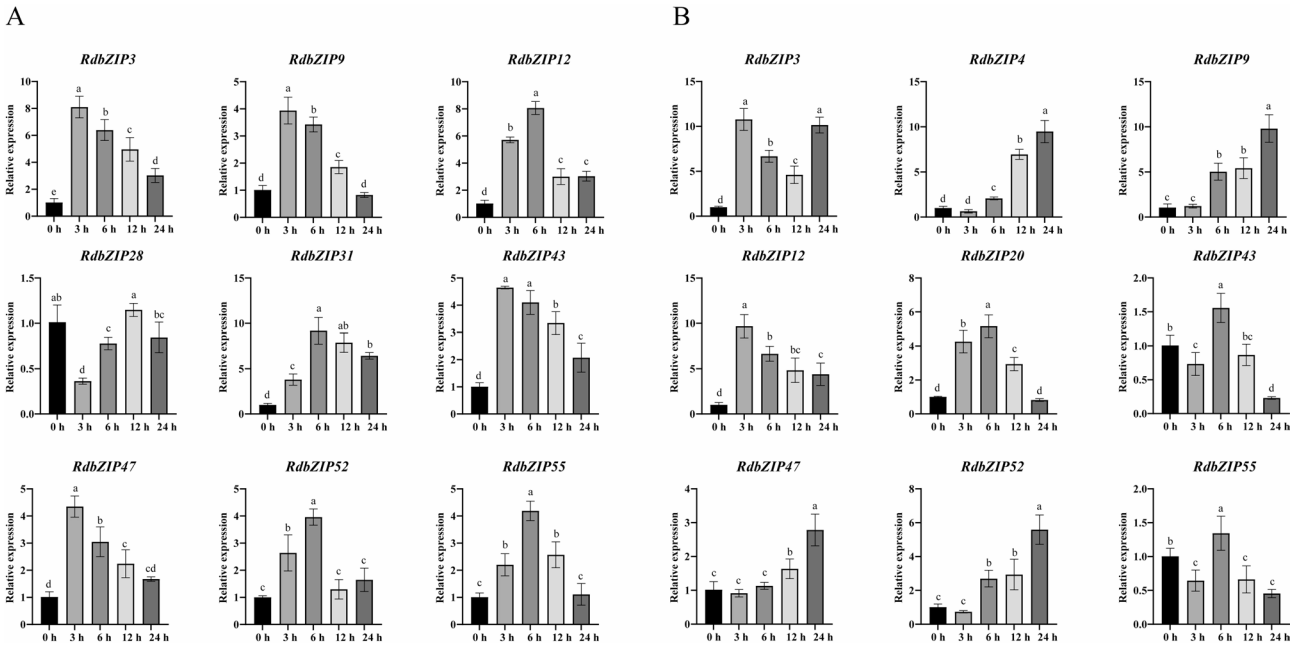

Fig. 8 Gene expression map of *RdbZIPs* under different abiotic stress. (**A**) 150 mM NaCl. (**B**) Drought (20% PEG 6000). Gene transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Different letters marked on the same bar chart indicate statistical significance of $p < 0.05$

patterns varied among subgroups: subgroups H and K exhibited collinearity with both *A. thaliana* and *R. chinensis*; subgroup C's *RdbZIP29* was colinear only with *AtbZIP*; subgroup J was colinear only with *RcbZIP*; and subgroup M showed no collinearity with either species. These findings suggest that *RdbZIP* genes may have experienced functional reshaping during evolution, and that the less numerous subgroups may possess unique roles, warranting further investigation.

The varied organization of gene structures may indicate the evolutionary trajectories of different *bZIPs* [5, 6], while the number of introns influences the potential for plant adaptation and developmental processes. In the *RdbZIP* family, the number of introns ranges from 0 to 22 (Fig. 5); specifically, the four genes in the S subfamily have 0 introns, whereas five genes possess 1 intron. Furthermore, the A, B, H, E, and I subfamilies contain relatively few genes with short introns or no introns that have been activated through evolutionary selection, such as *RdbZIP5*, *RdbZIP21*, and *RdbZIP55*. Notably, the *RdbZIP18* in the K subfamily presented a greater number of introns than did the other subfamilies. Research on rice indicates that the rate of intron acquisition following segmental duplication is slower than the rate of intron loss [4], suggesting that members of the K subfamily may represent more primitive genes. Each subfamily has a similar composition of conserved motifs. Subfamily A is involved in the plant ABA signaling pathway, regulating downstream gene expression and subsequently influencing plant growth, development, and responses to adverse conditions [12, 15].

Studies have exhibited that bZIP transcription factors are integral to numerous biological processes in plants and play a significant regulatory role in plant resistance to various stressors [15, 17–20, 57]. In this study, we examined the *cis*-elements in the promoters of these *RdbZIPs*, of which approximately one-third (458 of 1544 in total, Table S5) of these elements is associated to stress response. Moreover, these element types response for stress, particularly the ARE element (anaerobic response elements), were widespread across these *RdbZIPs*. Similar results were observed in *bZIPs* of wax gourd [50] and poplar [31]. The gene relative expression of 11 randomly selected *RdbZIPs* were significantly varied under high salt stress or drought stress, indicating that these *RdbZIPs* involved in response to stress. Additionally, the gene expression levels of *RdbZIP47* in the I subfamily, a homolog of *AtbZIP51* (AT1G43700), exhibited a tendency to rise and then fall with prolonged treatment time under salt stress, while its expression gradually increased under drought stress. These contrasting expression patterns suggest that *RdbZIP47* may play multiple roles in response to different stresses. However, the current study lacks direct functional evidence linking RdbZIP47 to stress tolerance., such as knockout or overexpression assays. Therefore, further mechanistic studies and functional validations are necessary to clarify the specific roles of *RdbZIP47* under various stress conditions. A recent study demonstrated that *AtbZIP51* (VIP1) and its homologs upregulate genes can enhance mechanical stress tolerance in leaves via AGCTG (G/T) and influence CAMTA-dependent gene expression [58]. However, the

regulatory mechanism of *RdbZIP47* in response to salt or drought stress requires further experimental verification.

Conclusion

In the present study, the *bZIP* gene family was comprehensively determined in the *R. delavayi*, and these genes features, including gene structure, evolutionary relationship, collinear-gene pairs, *cis*-acting elements, and potential functions in response to stresses were systematically analyzed. Herein, a total of 59 *RdbZIPs* were detected in in the *R. delavayi*, which were clustered into 13 subfamilies. The motifs and gene structures of these *RdbZIP* members were largely variable between any two subfamilies but were highly conserved within subfamily. The analysis of cis-acting elements revealed that most of these genes contained elements associated with hormone responses, growth and development, and stress responses. qRT−PCR analysis showed that the expression levels of most selected *RdbZIPs* were significantly upregulated or downregulated under drought and high-salt stress, indicating that *RdbZIPs* play important roles in the response of *R. delavayi* to different abiotic stresses.

Supplementary Information

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Supplementary Material 1

Acknowledgements
Not available.

Author contributions
M. C. wrote the manuscript and conducted the experiments. J. Y. and C. S. analyzed the data and conducted the bioinformatics analysis. J. O. provided experimental materials. J. O. and B. Z. contributed to the study of concepts and designs. All the authors read and approved the final manuscript.

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Data availability
The dataset analyzed during the current study, including the gene expression data from various tissues mentioned in the manuscript, is available in the National Center for Biotechnology Information (NCBI) repository, with the dataset accession number PRJNA907866 (https://www.ncbi.nlm.nih.gov/). The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Rhododendron delavayi seedlings were collected from the Baili Rhododendron Nature Reserve in Guizhou and were identified by Professor Jing Ou. The methods involved in this study were carried out in compliance with local and national regulations.

Competing interests
The authors declare no competing interests.

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